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The Folic Acid Requirement of Chicks for Growth, Feathering and Hemoglobin Formation.*

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Pfiffner and associates[1] isolated vitamin B_c (folic acid) in crystalline form from liver, and observed that when this substance was fed to chicks at the level of 250 μg per 100 g of deficient diet, they grew normally and exhibited no anemia at 4 weeks of age. Campbell, Brown, and Emmett[2] found that a vitamin B_c-deficient diet caused poor feath-

* The research work described in this paper has been undertaken in cooperation with the Quartermaster Corps Committee on Food Research and has been supported in part by grants from Cerophyl Laboratories, Inc., Kansas City, Mo., Lederle Laboratories, Inc., Pearl River, N.Y., and The Nutrition Foundation, Inc., New York, N.Y.

1 Pfiffner, J. J., Binkley, S. B., Bloom, E. S.,

ering, retardation in growth, and a macrocytic, hypochromic anemia. Some improvement in growth was provided by 20 μg of vitamin B_c, but 100 μg were necessary for normal growth. They stated that 40 μg of vitamin B_c completely corrected the hemoglobin level, although their data indicated that even the 100 μg level was not equivalent to the positive controls.

The same authors in a subsequent paper[3] found that by injecting vitamin B_c the same responses were obtained as when the factor was given orally. They concluded that the intestinal flora was not affected by folic acid, directly at least, but that the effect on growth, feathering, and blood cell components was caused by vitamin B_c *per se*. From the data of Pfiffner and associates[4] it appears that 25 μg of vitamin B_c per 100 g of diet were adequate for growth, but more than 25 μg were needed for maximum hemoglobin levels. Campbell, McCabe, Brown, and Emmett,[5] while studying the relationship of vitamin B_c to the cellular elements of chick blood, concluded that 20 to 200 μg of vitamin B_c were needed to maintain normal hematopoiesis in the growing chick for the first 4 weeks of life.

Briggs, Luckey, Elvehjem, and Hart[6] used the fermentation *L. casei* factor of Hutchings, Stokstad, Bohonos, and Slobodkin[7] at levels of 5, 20, and 50 μg per 100 g of diet. Succinylsulfathiazole, added to the 20 μg level of *L. casei* factor, was found to decrease the growth response about 60 g and the hemoglobin level 0.9 g per 100 cc of blood at 4 weeks of age. Their data indicated that 50 μg of *L. casei* factor were not adequate for normal growth and hemoglobin formation. This finding has been substantiated in this laboratory by Charkey and associates[8] who suggest that between 50 and 100 μg of *L. casei* factor per 100 g of diet (probably closer to the lower value) are needed for optimum response from this factor.

Angier and associates[9] using synthetic *L. casei* factor (folic acid) found that 50 μg per 100 g of diet gave as good a response as did 100 μg, indicating that the requirement for this factor is not greater than 50 μg.

Petering, Marvel, Glausier, and Waddell,[10] using a yeast extract and a liver fraction to supply vitamin B_c, observed complete prevention of deficiency symptoms when the amounts of the vitamin present were 22 and 44 μg per 100 g of diet, respectively. They concluded that the level of vitamin B_c required by the chick is probably less than 50 μg per 100 g.

Adequate data have been reported to establish the qualitative role of folic acid in promoting normal growth and hemoglobin formation in the chick and to suggest its relationship to normal feathering. In this paper 3 experiments are described in which the quantitative folic acid requirements of chicks for growth, feathering, and hemoglobin formation were determined.

Experimental. Single Comb White Leghorn cockerels, 15 to a lot, were housed in electrically-heated batteries placed in a room where the temperature was thermostatically controlled. All chicks were on wire mesh floors, and feed and water were supplied *ad libitum*.

The chicks were fed purified diet 653 as

Brown, R. A., Bird, O. D., Emmett, A. D., Hogan, A. G., and O'Dell, B. L., *Science*, 1943, **97**, 404.

2 Campbell, C. J., Brown, R. A., and Emmett, A. D., *J. Biol. Chem.*, 1944, **152**, 483.

3 Campbell, C. J., Brown, R. A., and Emmett, A. D., *J. Biol. Chem.*, 1944, **154**, 721.

4 Pfiffner, J. J., Calkins, D. G., O'Dell, B. L., Bloom, E. S., Brown, R. A., Campbell, C. J., and Bird, O. D., *Science*, 1945, **102**, 228.

5 Campbell, C. J., McCabe, M. M., Brown, R. A., and Emmett, A. D., *Am. J. Physiol.*, 1945, **144**, 348.

6 Briggs, G. M., Jr., Luckey, T. D., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1945, **158**, 303.

7 Hutchings, B. L., Stokstad, E. L. R., Bohonos, N., and Slobodkin, N. H., *Science*, 1944, **99**, 371.

8 Charkey, L. W., Daniel, L. J., Farmer, F. A., Norris, L. C., and Heuser, G. F., manuscript in preparation, Cornell University, Ithaca, New York.

9 Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., Semb, J., Stokstad, E. L. R., SubbaRow, Y., Waller, C. W., Cosulich, D. B., Fahrenbach, M. J., Hultquist, M. E., Kuh, E., Northey, E. H., Seeger, D. R., Sickels, J. P., and Smith, J. M., Jr., *Science*, 1945, **102**, 227.

10 Petering, H. G., Marvel, J. P., Glausier, C. E., Jr., and Waddell, J., *J. Biol. Chem.*, 1946, **162**, 477.

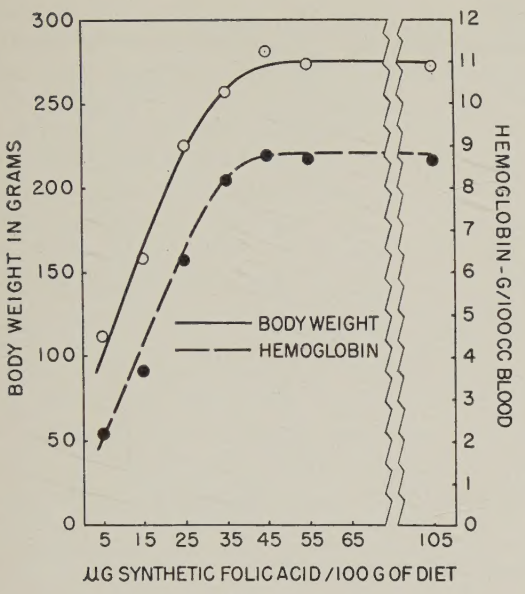

Fig. 1.
Growth and hemoglobin responses of chicks 4 weeks of age to synthetic folic acid. Each point represents the average of 75 chicks, except at the 5 μg and 105 μg points which are the averages of 30 chicks.

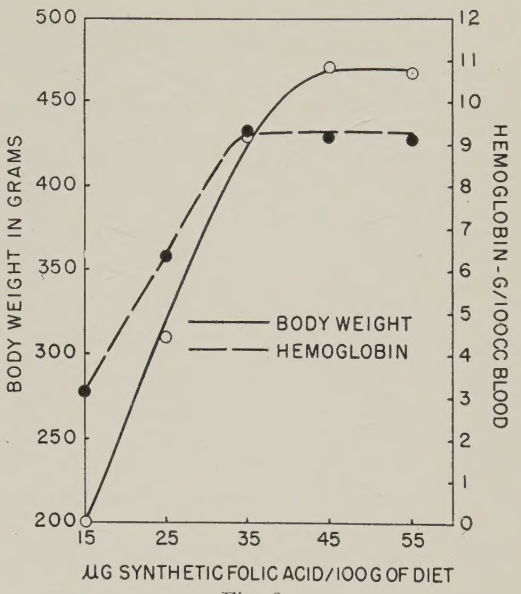

Fig. 2.
Growth and hemoglobin responses of chicks 6 weeks of age to synthetic folic acid. Each point represents the average of 45 chicks.

reported by Hill, Norris, and Heuser[11] plus a factor S concentrate equivalent to 5% yeast.† In Experiment 1, in which 50 μg of 2-methyl-3-hydroxy-4-carboxy-5-hydroxymethlypyridine (B-pyracin) were added per 100 g of diet, the basal diet was found to contain 5 μg of folic acid per 100 g by microbiological assay following chick liver incubation. In Experiment 2 the same amount of B-pyracin was added and the inositol level was reduced to 50 mg per 100 g of diet. No B-pyracin was included in the basal diet of Experiment 3. The folic acid content of the basal diets used in Experiments 2 and 3 was 15 μg per 100 g. The difference between the folic acid contents of the diets used is due to the factor S preparation added in Experiment 1 having only one-third the amount of folic acid as the preparations added in Experiments 2 and 3. The preformed folic acid in all basal diets was approximately 2.0 μg per 100 g.

Experiment 1 was divided into 2 parts: (a) folic acid fed at levels of 10, 20, 30, 40, 50, and 100 μg per 100 g of diet; and (b) folic acid at these same levels plus 1% succinylsulfathiazole. In Experiment 2 the supplements were: (a) 10, 20, 30, and 40 μg of folic acid per 100 g of diet; and (b) folic acid at these same levels plus 2% succinylsulfathiazole. The design of Experiment 3 was the same as 2 (a). Levels greater than 40 μg per 100 g were not added to the basal diets fed in Experiments 2 and 3, because this amount of folic acid plus the 15 μg already present in the diet, exceeded the minimum amount found necessary in Experiment 1.

Body weights were recorded weekly, and the hemoglobin levels were determined at the 2d, 3d, and 4th weeks in Experiment 1, and in Experiments 2 and 3 at the 3d, 4th, 5th, and 6th weeks. Hemoglobin was determined by the oxyhemoglobin method using a Coleman spectrophotometer to read the color produced, according to a method de-

[11] Hill, F. W., Norris, L. C., and Heuser, G. F., *J. Nutrition*, 1944, **28**, 175.

† The authors are indebted to Anheuser-Busch, Inc., St. Louis, Mo., for Strain S dried brewers' yeast; to Lederle Laboratories, Inc., Pearl River, N.Y., for synthetic *L. casei* factor (folic acid); and to Merck and Co., Rahway, N.J., for crystalline B-pyracin.

TABLE I.
Results of Feeding Synthetic Folic Acid to Chicks for 6 Weeks.

Folic acid added per 100 g diet μg	Avg wt 4 wk g	Avg wt 6 wk g	Hemoglobin 4 wk g per 100 cc	Hemoglobin 6 wk g per 100 cc	Feather score 6 wk %
	Experiment 2 (a)				
None*	153 (11)†	202 (5)	3.9‡	2.4	25‡
10	225 (15)	345 (12)	5.1	6.9	37
20	249 (15)	412 (15)	8.0	9.5	77
30	288 (15)	476 (15)	8.3	9.2	83
40	274 (14)	461 (14)	8.7	9.3	98
	Experiment 2 (b) + 2% succinylsulfathiazole				
None	153 (8)	173 (2)	3.2	1.6	0
10	206 (12)	310 (11)	4.8	6.6	36
20	248 (14)	431 (14)	7.3	9.1	81
30	282 (14)	469 (14)	8.2	9.5	89
40	281 (14)	470 (14)	8.5	9.1	96

* Basal diet 653 + factor S concentrate equivalent to 5% yeast contained 15 μg of folic acid per 100 g.
† Numbers in parentheses indicate the number of surviving chicks.
‡ Hemoglobin values and feather scores were determined on all chicks.

veloped in this laboratory.

In Experiments 2 and 3 feathering was scored on the chicks at 6 weeks of age as follows: 4—excellent; 3—good; 2—fair; 1—poor; 0—very poor. Growth, smoothness and condition of the feathers were taken into consideration in determining feather score which was calculated for each lot by the formula:

$$\frac{\text{Sum of scores of individual chicks}}{4 \times \text{No. of chicks}} \times 100 = \text{degree of feathering (\%)}$$

Results. The data obtained at 4 weeks of age from the 3 experiments are presented in combined form in Fig. 1. In Experiments 1 and 2 the data obtained from the sulfonamide and non-sulfonamide diets were not significantly different. It was, therefore, considered justifiable to combine the results from all groups in determining the requirements.

From Fig. 1 it is evident that the amount of folic acid required for optimum growth and hemoglobin formation to 4 weeks of age under the experimental conditions is approximately 45 μg per 100 g of diet. A straight-line response was obtained in growth and hemoglobin levels from 5 μg to almost 45 μg of folic acid per 100 g of diet.

Data from Experiments 2 and 3 which show growth and hemoglobin response at 6 weeks of age are presented in Fig. 2. The requirement of folic acid for maximum growth was found to be 45 μg per 100 g of diet, the same as at 4 weeks of age. However, the requirement to maintain normal hemoglobin values was 35 μg per 100 g of diet, showing that as the chicks became older less folic acid was needed.

The results of Experiment 2 are presented in Table I. These data are included because this experiment is typical of those conducted to determine the folic acid requirement of the chick. In addition, the data demonstrate that succinylsulfathiazole had no effect on the response to folic acid under the conditions of the experiment, and that folic acid was directly concerned with feathering. Hemoglobin values and body weights at 4 and 6 weeks of age, and feather scores at 6 weeks of age on sulfonamide and non-sulfonamide diets are given.

Perosis occurred in each of the experiments. A report was made by Daniel, Farmer, and Norris[12] on the effect of folic acid on perosis. The perosis was more severe on the sulfonamide diet than on the non-sulfonamide diet. This was especially noticeable at the lower levels of folic acid. Because of

[12] Daniel, L. J., Farmer, F. A., and Norris, L. C., *J. Biol. Chem.*, 1946, **163**, 349.

the severity of perosis many of the chicks were unable to secure food and water and died before the end of the 6-week experimental period. As a result the 6-week weights and hemoglobin values of the first 2 lots on the sulfonamide diet are somewhat lower than the corresponding lots on the non-sulfonamide diet.

The feather scores indicated that succinylsulfathiazole included at 2% of the basal diet had no detrimental effect on feathering. The feather score in the basal lot of Experiment 2(b) was not considered typical since it represented the scores of only 2 chicks. There was no difference in the feather scores obtained on the 2 diets indicating that folic acid *per se* is concerned with feathering and that intestinal synthesis of a feather factor is not probable. The requirement of folic acid for good feather development appears to be not less than 55 μg per 100 g of diet.

Survival of chicks to 6 weeks of age in Experiment 2 required approximately 25 μg of folic acid per 100 g of diet. Data from Experiments 1 and 3 confirm this estimate.

Summary. The folic acid requirement of chicks for growth, feathering, and hemoglobin formation has been determined using a purified diet containing 2.0 μg of preformed folic acid per 100 g of diet. The total folic acid content of the basal diets as determined microbiologically following incubation with chick liver was 5 μg per 100 g of diet for Experiment 1 and 15 μg for Experiments 2 and 3.

The following amounts of folic acid, which include the amount in the basal diet, were required for its several functions: for survival to 6 weeks of age, approximately 25 μg per 100 g; for growth and hemoglobin formation at 4 weeks of age, 45 μg; for growth at 6 weeks, 45 μg, but for hemoglobin formation at 6 weeks, 35 μg; and for feathering at 6 weeks of age, not less than 55 μg per 100 g of diet.

In these experiments the addition of succinylsulfathiazole at 1 or 2% of the diet did not have any effect on growth, feathering, or hemoglobin formation in chicks to 6 weeks of age.

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Milk as a Source of the Monkey Anti-Anemia Factor.*

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Previous studies[1,2] have shown that whole liver powder, raw and lyophilized liver are good sources of the monkey anti-anemia factor. In this paper we wish to report experiments carried out to determine if milk contains this factor.

Experimental. The method of handling the monkeys has been described previously.[3] The assay monkeys consisted of animals which failed to show complete recovery from a riboflavin deficiency after riboflavin therapy,[2] and animals made deficient in the monkey anti-anemia factor by the prolonged feed-

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E. M. Sporn gave us helpful assistance in the work.

1 Cooperman, J. M., Waisman, H. A., McCall, K. B., and Elvehjem, C. A., *J. Nutrition*, 1945, **30**, 45.

2 Cooperman, J. M., McCall, K. B., and Elvehjem, C. A., *Science*, 1945, **102**, 645.

3 Waisman, H. A., Rasmussen, A. F., Jr. Elvehjem, C. A., and Clark, P. F., *J. Nutrition*, 1943, **26**, 205.

TABLE I.

Response in Weight, Hemoglobin and Differential Leucocyte Count of Monkeys Deficient in the Anti-anemia Factor to Milk and Milk Preparations.

Monkey No.	Treatment	Wt (g)		Hemoglobin (g/100 cc)		Differential leucocyte count Before		After 3 wk	
		Before	After 3 wk	Before	After 3 wk	% neutrophiles	% lymphocytes	% neutrophiles	% lymphocytes
186	Raw whole milk	3140	3460	12.77	13.66	63	30	21	62
217	" " "	4000	4200	12.59	14.97	59	27	25	67
82	Pasteurized whole milk	3420	3620	10.43	13.33	48	44	26	60
276	Pasteurized whole milk	2230	2300	12.77	13.56	50	37	25	64
248	Raw cream	2800	2910	12.60	13.11	49	42	39	55
277	" skim milk	2900	3240	11.61	13.71	62	28	30	65
189	" whey	3600	3900	12.41	13.77	80	20	17	72

ing of a corn grits basal diet.[4] All of these monkeys showed poor weight gains, suboptimal hemoglobin levels, and reversed neutrophile-lymphocyte ratios, the typical syndrome in a deficiency of the monkey anti-anemia factor. If allowed to continue on the assay rations without further treatment, the animals become progressively worse and die within 2 months. The relative potency of the materials to be tested was determined by the response in weight, hemoglobin and differential leucocyte count observed in the monkey during a 3-week period. The milk and milk derivatives were given together with the vitamin supplements each day. In all trials at least one monkey on the corn grits diet and one on the riboflavin diet were used.

Results. Seven monkeys were given 50 cc of raw whole milk per day. The animals took this supplement very readily and the response of 2 typical monkeys, No. 186 and 217, is shown in Table I.

Two other monkeys, No. 82 and 276, were given 50 cc of pasteurized whole milk per day, and a definite response was elicited, although it was of a smaller magnitude than the response to raw whole milk (Table I.) This suggests that pasteurization causes some destruction of the monkey anti-anemia factor and verifies the earlier observations as to the lability of this factor.[1,2]

[4] Cooperman, J. M., McCall, K. B., Ruegamer, W. R., and Elvehjem, C. A., *Fed. Proc.*, 1946, **5**, 230.

Since Van Wagtendonk *et al.*[5] reported that raw cream contains a labile anti-stiffness factor essential for guinea pigs, the activity of raw cream for the monkey was determined. Monkeys 248 and 249 were given 10 cc of raw 35% cream. Two other monkeys, No. 277 and 166, were given 50 cc of raw skim milk per day. It is apparent from Table I that the larger portion of the material remained in the skim milk and that all of the activity of the cream could be accounted for by its skim milk content. It was thus evident that the monkey anti-anemia factor is distinct from the guinea pig anti-stiffness factor.

Since raw skim milk was apparently a good source of the monkey anti-anemia factor, further fractionation was attempted. Raw skim milk was treated with a commercial rennet preparation to coagulate the casein and then centrifuged. The clear whey was fed at a level of 50 cc per day to monkey 189. After one week on this regimen the monkey failed to show a significant weight gain. In all probability the casein had carried down some of the activity. However, when the level of whey was raised to 100 cc per day, the monkey started to gain at a rapid rate, as can be seen in Table I. Monkey 186 suddenly started to lose weight. It was then given 100 cc of raw whey per day and the weight loss was checked after one day, and during the next week the monkey gained weight rapidly.

[5] Van Wagtendonk, W. J., Schocken, V., and Wulzen, R., *Arch. Bioch.*, 1944, **3**, 305.

Summary. Raw whole milk is a good source of the monkey anti-anemia factor. Pasteurization of the milk seems to reduce the amount of this factor in milk to some extent. This factor can be differentiated from the guinea pig anti-stiffness factor by the fact that raw cream possesses little activity while the raw skim milk accounts for the greater portion of the activity in whole milk. Raw whey, made by enzymatic treatment of raw skim milk, is also a good source of the monkey anti-anemia factor.

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Effect of Adrenocorticotrophic Hormone on Urinary Nitrogen Excretion in the Normal Rat.*

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It is well recognized that adrenocorticotrophic hormone (ACTH) and adrenal cortical steroids inhibit normal body growth.[1-4] Retardation of chondrogenesis and osteogenesis in the region of the proximal epiphysis of the tibia has likewise been produced by ACTH and has been shown to be mediated by the adrenal.[5] In the hypophysectomized animal, ACTH has been shown to inhibit the growth produced by growth hormone.[6] Since it is generally recognized that nitrogen retention accompanies somatic growth, increased nitrogen excretion might be anticipated following the injection of ACTH. Such a finding is reported in this paper.

Normal, year-old, "plateaued" female rats of the Long-Evans strain weighing between 250 and 320 g were fed 12 g daily of a purified† diet which was completely consumed by all rats. Individual urines were collected at 24-hour intervals. Total urinary nitrogen was determined by the micro-Kjeldahl method. The ACTH was prepared by the procedure described previously.[7,8] Following a control period of 24 days, 5 rats were injected subcutaneously with 1 mg of hormone 6 times daily for 6 days. Six control animals were similarly injected with the same amount of

* Aided by grants from the Research Board of the University of California and the Rockefeller Foundation. The following materials were generously contributed: crystalline B vitamins from Hoffmann-La Roche Company, Nutley, N.J., Lederle Laboratories, Inc., Pearl River, N.Y., and Merck and Company, Inc., Rahway, N.J.; alpha-tocopherol from Merck and Company, Inc., Rahway, N.J.; liver fraction powder (7-5293) from Lederle Laboratories, Inc., Pearl River, N.Y.

1 Moon, H. D., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 34.

2 Ingle, D. J., Higgins, S. M., and Kendall, E. C., *Anat. Rec.*, 1938, **71**, 363.

3 Wells, H. B., and Kendall, E. C., *Proc. Staff Meet. Mayo Clinic*, 1940, **15**, 324.

4 Evans, H. M., Simpson, M. E., and Li, C. H., *Endocrinology*, 1943, **33**, 237.

5 Becks, H., Simpson, M. E., Li, C. H., and Evans, H. M., *Endocrinology*, 1944, **34**, 305.

6 Marx, W., Simpson, M. E., Li, C. H., and Evans, H. M., *Endocrinology*, 1943, **33**, 102.

† The diet consisted of alcohol-extracted casein 24%, sucrose 63.5%, hydrogenated vegetable oil (Crisco) 8%, salts[11] 4%, and liver fraction powder 0.5%. Crystalline B vitamins are added per kilogram diet; thiamine HCl 5 mg, pyridoxine HCl 5 mg, riboflavin 10 mg, *p*-aminobenzoic acid 10 mg, nicotinic acid 20 mg, calcium pantothenate 50 mg, inositol 400 mg, and choline chloride 1 g. One cc of a fat-soluble vitamin mixture containing 6 mg alpha-tocopherol, 115 chick units vitamin D, 800 U.S.P. units vitamin A, and 650 mg corn oil (Mazola) is given weekly.

11 Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. B. C.*, 1944, **138**, 459.

7 Li, C. H., Simpson, M. E., and Evans, H. M., *Science*, 1942, **96**, 450.

8 Li, C. H., Evans, H. M., and Simpson, M. E., *J. B. C.*, 1943, **149**, 413.

Opposing Effects of ACTH and GH on Body Weight and Urinary Nitrogen Excretion of Pair-Fed Plateaued Female Rats

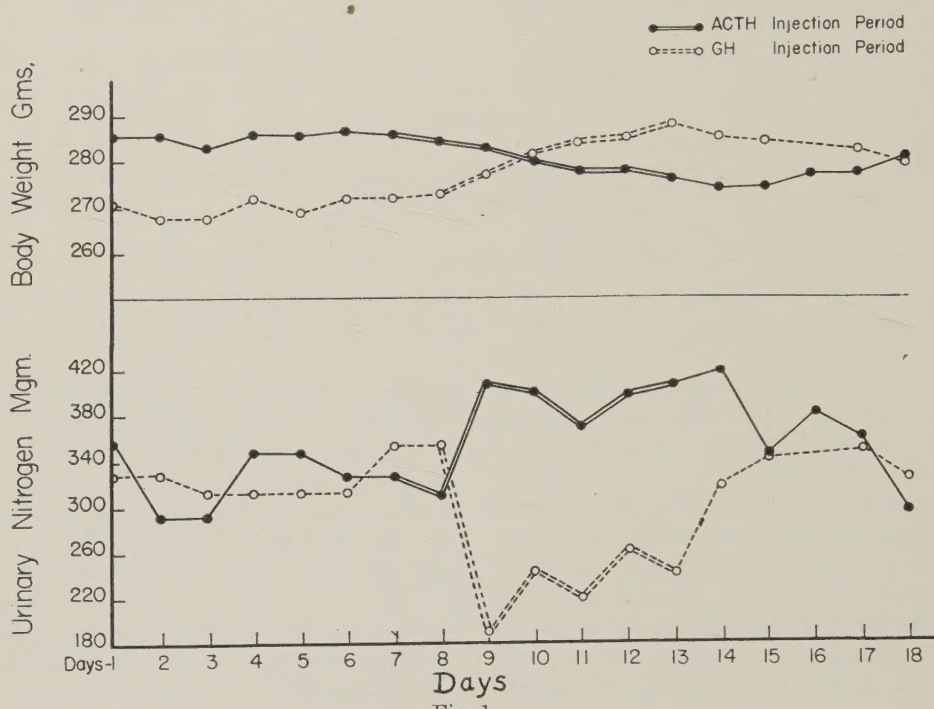

Fig. 1.

protein derived from crude anterior pituitary gonadotrophic extract, inactivated by boiling.

In the 10 days prior to injection, the daily nitrogen excretion per rat was 326 ± 7.8‡ mg and 321 ± 12.4 mg for the experimental and control group respectively. In the first 24 hours of ACTH injection, no change in nitrogen excretion occurred. During the last 5 days of ACTH injection, the daily excretion increased to 394 ± 8.0 mg, an increase of 20.8%, and this increase persisted in the 24-hour period after the injections were stopped. This increase was found to be statistically significant, having a p value of 0.01.[9] Twenty-four hours after stopping ACTH the excretion fell to 343 ± 9 mg, approximately the preinjection level.

In the control group, the daily nitrogen excretion, which had been 321 ± 12.4 mg prior to injection, rose to 352 ± 8.0 mg, an increase of 8.8%, during the injection period. This increase was found not to be highly significant, having a p value of .035.

Decrease in body weight occurred concomitantly and proportionately to the increased nitrogen excretion.§ In the experi-

‡ Mean ± standard error.

9 Fisher, R. A., *Statistical Methods for Research Works*, 6th ed., Edinburgh, London, Oliver and Boyd, 1936.

§ The urines of experimental animals were also examined for reducing substances by the Somogyi method. Glycosuria developed in but one rat on the first, second, and sixth days of the injection period. It may be recalled that Ingle *et al.*[10] have induced glycosuria in normal rats with ACTH using a diet containing 15 g of available carbohydrate per day. The discrepancy may therefore be attributed to the difference in experimental conditions.

10 Ingle, D. J., Winter, H. A., Li, C. H., and Evans, H. M., *Science*, 1945, **101**, 671.

mental group, the average weight prior to injection was 286 g. At the end of the injection period, the weight had decreased to 276 g, and continued to fall to 274 g during the subsequent 24-hour period of continued nitrogen excretion. In the control group, on the contrary, the weight remained constant at 283 g.

For contrast, the data are presented in Fig. 1 for this and a similar experiment using purified growth hormone (GH) 0.5 mg intraperitoneally twice daily. On this graph the opposing effects of ACTH and GH on body weight and urinary nitrogen excretion are apparent.

Summary. ACTH causes an increase in urinary nitrogen excretion with proportionate loss of body weight in the normal rat. The effect of subcutaneous ACTH on nitrogen excretion is manifest on the second day and persists 24 hours after injection is stopped.

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Growth of Japanese B Encephalitic Virus in the Yolk of the Developing Egg.

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In an endeavor to produce a chick-embryo vaccine against the Nakayama strain* of Japanese B encephalitic virus, experiments were undertaken to determine how to develop a more potent virus in the egg. Haagen and Crodel[1] reported the cultivation of this virus on the chorio-allantoic membranes of the developing chick, while Smith and Lennette[2] later reported growth on the membranes of 10- to 13-day-old embryonated eggs. The titers in mice seldom were above 10^{-3}. Sabin[3] and his associates in the investigation of vaccine production considered the use of chick-embryo tissues as unsuitable because of the low potency obtained after inoculation either into the yolk or the allantoic fluid. Since they did not mention the titers obtained nor give any details of the egg work, it was thought of interest to investigate further what potency could be developed in the egg by either the allantoic or the yolk sac routes.

Allantoic route. Ten- to 11-day-old embryonated eggs were inoculated with 0.2 cc of a 10% mouse brain suspension through the air sac membrane into the allantoic fluid. To determine the most suitable incubation period for optimum virus growth, eggs were opened in 24, 48, 72, and 96 hours, respectively, after inoculation. Twenty percent suspensions of the embryos and chorio-allantoic membranes in buffered saline were prepared by grinding in a small Waring blendor for 2 minutes. After reduction to a 10% suspension, each material was titrated intracerebrally in mice in 0.03 cc amounts and the 50% endpoint obtained according to the method of Reed and Muench.[4] With the exception of a low titer ($10^{-3.74}$) in 24 hours, the other endpoints remained fairly constant—$10^{-6.2}$ to $10^{-6.5}$ and $10^{-6.74}$. Most of the embryos were alive, except on the fourth day.

Live embryos taken on the third day of incubation were then used for passage material, 16 serial passages being made in this

* The Nakayama strain was kindly sent from the Department of Virus Research at Lederle Laboratories.

1 Haagen, E., and Crodel, B., *Zentralbl. f. Bakt.*, 1938, **142**, 269.

2 Smith, M. G., and Lennette, E. H., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 323.

3 Sabin, A. B., assisted by Duffy, C. E., Warren, J., Ward, R., Peck, J. L., Jr., and Ruchman, I., *J. Am. Med. Assn.*, 1943, **122**, 477.

4 Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

TABLE I.
The Virus Concentrations Obtained by Varying the Age of the Eggs and Keeping the Inoculum Constant Using (1) Embryos and Membranes and (2) Yolk Sacs.

Age of eggs in days	Amt of virus	Titers in mice 50% endpoint of virus (log. exponent)
	cc	
7	0.2	7.50
		6.50
8	,,	7.00
		6.50
9	,,	7.24
		5.74
10	,,	6.50
		5.24

manner, using 0.2 cc of a 10% suspension of embryo and chorio-allantoic membranes in buffered saline. The embryos and membranes were tested for potency in mice at different intervals through the 13th passage. The titers were again quite constant, varying from $10^{-6.50}$ to $10^{-6.74}$, with only one jump to $10^{-7.24}$ on the fifth passage and a drop to $10^{-6.0}$ on the 13th.

At no time was the potency equal to that of the mouse brains used in the initial inoculum ($10^{-8.33}$). Repeated egg passages failed to increase the virulence and the lethal effect was quite inconstant. Occasionally embryos would die on the third or fourth days, but more often all remained alive. The eggs were not incubated longer because of the development of feathers and the greater danger of an allergic reaction from the older embryonic tissues if they were used for vaccine production.

Yolk sac route. It was then decided to inoculate younger eggs directly into the yolk after the method of Cox[5] for Rocky Mountain spotted fever and epidemic typhus rickettsiae. The first trial inoculations, using 0.2 cc of a 20% suspension of mouse brain virus into 8-day-old eggs, resulted in 100% mortality of the embryos within 48 hours of incubation. This lethal effect was noticed after repeated inoculations into eggs not only after direct injection of virulent mouse brain but after serial passage. Bacterial cultures were made on all tissues removed and as only bacteria-free material was used for egg inoculations, the high mortality was apparently due to the virus.

Since death of the embryo occurred regularly by this method of yolk inoculation, the lethal effect could now be used for various experiments as well as for titrating the virus in eggs instead of mice, as heretofore. In fact, this method proved to be more delicate for determining the potency of the virus than did the intracerebral titration in mice. A suspension of the embryos and membranes of the fifth yolk passage in eggs had a titer in eggs of $10^{-7.66}$ and only $10^{-6.74}$ in mice. On another occasion the tissues were lethal in eggs to $10^{-7.24}$. However, the usual titer of the mouse brain virus was $10^{-8.5}$ or better.

Experiments to determine the highest virus concentration in eggs by varying different factors:

(a) *Keeping a constant amount of inoculum and varying the age of the egg.* 0.2 cc of 10% mouse brain virus (50% endpoint $10^{-8.5}$) in buffered saline was inoculated into the yolk of 7-, 8-, 9-, and 10-day-old eggs, respectively. There were 6 eggs of each age. All of the embryos were dead within 48 hours, except those initially 10 days old, which died after 72 hours incubation. The embryos, chorio-allantoic membranes, and yolk sacs were removed from each set of eggs. The first 2 substances were ground together and the other separately to a 20% suspension in buffered saline, diluted to 10% and titrated in tenfold dilutions in 11 g mice, using dextrose-saline and 10% normal rabbit serum as diluent. This holds true for the experiments given in Tables I, II, III, and IV.

As seen in Table I, there was not much difference in the titers of virus in the embryos and membranes from the 7-, 8-, or 9-day-old eggs, but there was decidedly less virus present in the yolk sacs. The tissues of the 10-day-old embryos were the least potent.

(b) *Varying the amount of mouse brain inoculum and keeping the age of the egg constant.* Eight-day-old eggs were inoculated into the yolk with varying amounts of 10% mouse brain virus, allowing 4 eggs to each dilution. All of the embryos, regardless of

5 Cox, H. R., *Pub. Health Rep.*, 1939, **53**, 2241.

TABLE II.
The Virus Concentrations Obtained by Varying the Inoculum and Keeping the Age of the Egg Constant.

Age of egg	Amt of 10% mouse brain inoculum into yolk	Titers in mice of embryos and membranes, 50% endpoint of virus (log. exponent)
	cc	
8 days	0.05	6.74
	0.10	6.74
	0.20	7.00
	0.30	6.74
	0.50	7.24
	0.50	7.50
	1.00	7.0
	1.00	7.54

the amount given, were dead within 48 hours. The results of the titrations of the combined embryos and membranes are given in Table II.

The titers of the virus in the embryos and membranes increased as the amount of the inoculum increased. The highest titer was $10^{-7.5}$ while the original mouse brain inoculum was $10^{-8.5}$.

(c) *Keeping the amount of mouse brain virus and the age of the egg constant but varying the incubation period.* Eight-day-old eggs were inoculated via the yolk sac route with 0.2 cc of high titer mouse brain virus. Two of them were removed, respectively, at different time intervals, and the amount of virus present in the embryos and membranes was determined by titration in mice as shown in Table III.

The embryos were alive through 36 hours of incubation but all those remaining died within the 48 hours. The highest titer was obtained in the embryos and membranes removed after 36 hours of incubation when they were still active. There was a tenfold drop in titer in the dead embryos.

TABLE III.
The Virus Concentrations Obtained by Keeping the Inoculum and the Age of the Egg Constant but Varying the Incubation Period After Inoculation.

Hr of incubation	Titers in mice of embryos and membranes, 50% endpoint of virus (log. exponent)
6	4.00
12	3.50
18	6.50
24	7.50
36	8.24
48	7.24

(d) *To determine any increase in potency of the virus by serial passage in eggs.* Serial passages of 20% suspensions of embryos and membranes diluted in buffered saline were made by the yolk sac method in 8-day-old eggs. All embryos died in 48 hours and the tissues were tested for potency by intracerebral inoculation of mice weighing 10 to 11 g. The results are given in Table IV.

The titers in mice of the different egg passages remained fairly constant, although there was an increase for the ninth passage in this experiment. Usually the first passage showed the most virus, the titer depending on the strength of the original mouse brain virus.

(e) *Effect of temperature on the egg cultures.* Five different groups of 10-day-old eggs were inoculated via the allantoic fluid on different occasions and represented first, second, and third egg passages. One-half of each group was incubated at 95°F and the other half at 98°F. The embryos and chorioallantoic membranes were harvested on the third day of incubation and then tested for potency in mice. Invariably the highest titers occurred in the tissues incubated at 95°F.

Several groups of 8-day-old eggs were inoculated via the yolk sac method and harvested after the death of the embryos in two days. Part of these eggs had been incubated at 95°F and the remainder at 98°F. Once again the titers in mice ran slightly higher for those embryos placed at the lower temperature.

TABLE IV.
The Virus Concentrations Obtained After Serial Passage in Eggs.

No. of passages in eggs	Titers in mice of embryos and membranes, 50% endpoint of virus (log. exponent)
1	7.24
2	6.50
3	7.24
4	7.00
5	6.74
6	6.74
9	7.50

For this reason all eggs used in these experiments with the Japanese B virus were placed at 95°F.

(f) *Use of rabbit serum in the diluent.* The results of these experiments had all been obtained when the chick embryos were ground in buffered saline. To determine if the addition of inactivated normal rabbit serum to the diluent would produce more potent virus, the following experiment was undertaken:

Eight-day-old eggs were inoculated into the yolk with 0.2 cc, 0.5 cc, and 1.0 cc, respectively, of a 10% mouse brain suspension of virus ground in dextrose-saline containing 50% normal rabbit serum. All of the embryos died within 48 hours. The harvested embryos and membranes gave titers in mice of $10^{-7.5}$, $10^{-7.74}$, $10^{-7.0}$, respectively, in the order of the doses given above. The inoculation of 0.5 cc of virus yielded the most potent product. However, while the titers obtained were fairly high, equal values had likewise resulted when the diluent was only buffered saline. Therefore, any slight increase in potency by addition of rabbit serum would not offset the disadvantage of a possible carrying over of another foreign protein into any prospective vaccine.

Discussion. Since this work was submitted for publication, Warren and Hough[6] have reported on the successful use of a vaccine against the Japanese B virus prepared from infected chick embryos. They were able to obtain slightly higher titers of the virus by inoculation into the allantoic fluid than shown here. They likewise found that the potency increased after serial passage and that the embryos died after 4 days incubation.

[6] Warren, J., and Hough, R. G., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 109.

Perhaps their higher values may have been influenced by the addition of normal chicken serum to the inoculum and the lethal effect may have been due to chance inoculation of the yolk when using the younger (7-8-day-old) eggs for the allantoic route.

In the present work serum had not been added to any inoculum, except for one experiment, in order to avoid that complicating factor. Likewise when inoculating into the allantoic fluid, 10-11-day-old eggs had been employed because the surface area of the chorio-allantoic membranes was greater than in the younger eggs and there was less chance of injecting into the yolk. However, the lethal effect was both irregular and prolonged when using the allantoic route in the older eggs, so that it was a satisfaction to find that the yolk sac method in 7-8-day-old eggs gave quicker and more clear-cut results.

Summary. From the results of these experiments to develop a more potent virus in the egg, it is concluded that the greatest amount of virus of the Japanese Nakayama strain of encephalitis would probably be obtained in chick embryos by using 7- or 8-day-old embryonated eggs inoculated into the yolk with 0.5 cc amounts of mouse brain virus of a potency of $10^{-8.5}$ or better, and incubating 36 hours at 95° to 96°F. The use of normal rabbit serum in the diluent might raise the potency slightly, but would be contra-indicated if there was a possibility of carrying over another foreign protein into any prospective vaccine. Because of the constant lethal effect, inoculation of the egg yolk is of value for titration of this virus and for use in other quantitative experiments. At no time, however, did the titrations of egg tissue reach as high endpoints as those obtained with the mouse brain virus.

15388

Treatment of Experimental Syphilis in Rabbits with Streptomycin.

Sture A. M. Johnson and John D. Adcock. (Introduced by C. C. Sturgis).

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Streptomycin, discovered by Schatz, Bugie, and Waksman,[1] was shown by Heilman[2] to have an effect on experimental relapsing fever and leptospirosis. For this reason the present preliminary study was undertaken to determine the effect of streptomycin on the course of experimental syphilis in the rabbit.

Procedure and results. Three white, adult, male rabbits were inoculated intratesticularly with darkfield positive testicular material obtained from a rabbit previously infected with the Nichols' strain of *Spirocheta pallida.* All 3 rabbits so inoculated developed darkfield positive lesions within 23 days. Repeat darkfield examinations of the same 3 rabbits were still positive 24 days later. The quantitative Kahn reactions varied from 20 to 160 units. At this time 2 of the rabbits (No. 244 and No. 247) were weighed* and streptomycin therapy was started. Each of these 2 rabbits received 30 mg (30,000 units) of streptomycin† at 8:00 A.M., 12:00 M., 4:00 P.M., 8:00 P.M. and 80 mg at 12:00 midnight, making a total of 200 mg of streptomycin daily. The material was injected into the deep muscles of the back. After 24 hours of therapy one testicle of rabbit No. 244 was removed and found to contain motile spirochetes by darkfield examination. The quantitative Kahn reaction was 20 units. A normal saline emulsion was made of the testicle and injected intratesticularly into a normal rabbit which developed darkfield positive lesions in 40 days, with a Kahn reaction of 40 units.

The dosage schedule was continued unchanged for both rabbits and at the end of 48 hours of therapy one testicle of the second rabbit (No. 247) was removed. Darkfield examination revealed motile spirochetes. The quantitative Kahn reaction was 160 units. Again a testicular emulsion was prepared and inoculated into another rabbit which developed darkfield positive lesions in 40 days with a Kahn reaction of 320 units. No change in the dosage schedule was made and at the end of 72 hours of treatment, the remaining testicle of rabbit No. 244 was removed and therapy was discontinued in this animal. No spirochetes were seen on darkfield examination of this testicle. The quantitative Kahn reaction was 40 units. A testicular emulsion was prepared and inoculated into 2 rabbits. One rabbit failed to show clinical or serological evidence of syphilis in 94 days when it died. The second rabbit failed to show evidence of syphilis in 137 days. It was later successfully inoculated intratesticularly with darkfield positive rabbit material. Treatment was continued to rabbit No. 247 for a total of 96 hours. At that time, the remaining testicle was removed. There were 6 non-motile spirochetes per 100 darkfields. The quantitative Kahn reaction was 400 units. An emulsion of the testicle was inoculated into 2 rabbits. Both of these rabbits developed darkfield positive lesions, which did not appear, however, until 120 days had elapsed. The quantitative Kahn

1 Schatz, A., Bugie, E., and Waksman, S. A., Proc. Soc. Exp. Biol. and Med., 1944, **55**, 66.

2 Heilman, F. R., *Proc. Staff Meet., The Mayo Clinic,* 1945, **20**, 169.

* Rabbit No. 244 weighed 4.7 kg and No. 247 weighed 4.4 kg.

† Amounts of streptomycin are reported as equivalents of pure streptomycin base according to the recently adopted plan whereby 1 unit equals 1.0 μg of pure base. The streptomycin used in these experiments was furnished by Merck and Company, Inc., Rahway, N.J. The material was supplied as a partially purified dry powder containing the equivalent of 150 μg of pure streptomycin base per mg of solid. It was dissolved in sterile distilled water so that each cubic centimeter contained the equivalent of 50 mg of streptomycin base.

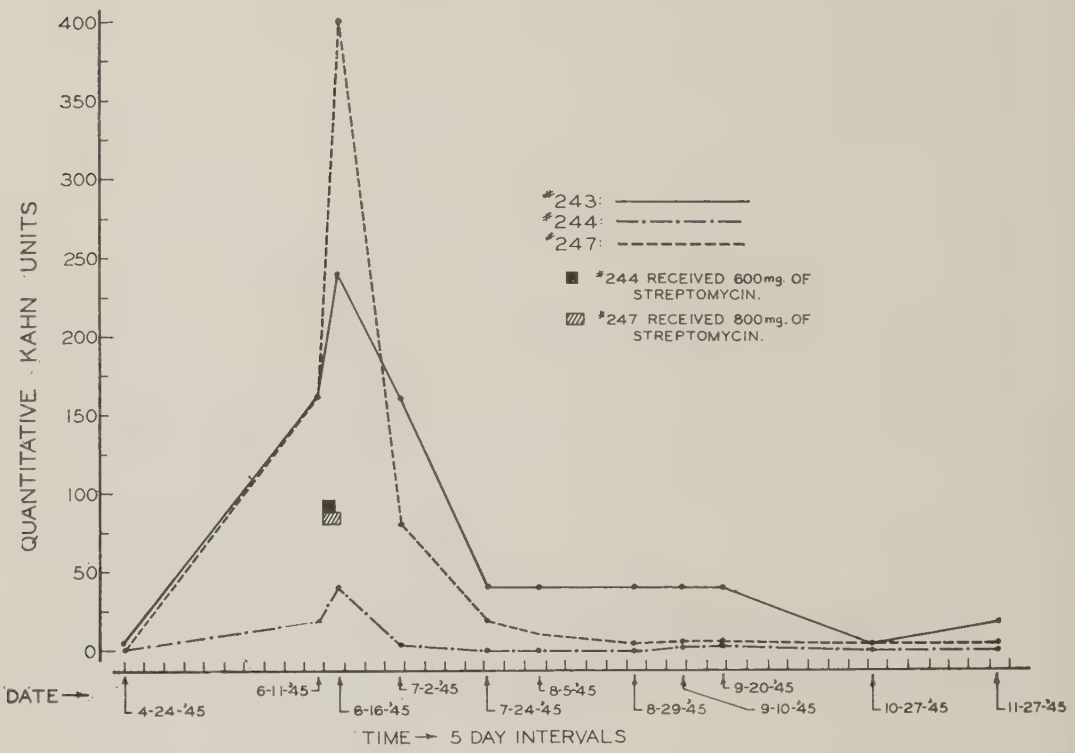

Fig. 1.
The results of the quantitative Kahn reactions on the blood of the treated and control rabbits.

reactions were 20 and 60 units respectively. At the time of removal of the second testicle of rabbit No. 247, both testicles of the third rabbit No. 243, which served as a control, were removed. An emulsion of one of these 2 testicles caused darkfield positive testicular lesions in a recipient rabbit 40 days later. Repeated quantitative Kahn reactions were done on the 2 streptomycin treated rabbits and on the control. The results are shown in Fig. 1.

Six months following treatment the popliteal nodes of the original 3 rabbits (No. 243, 244, and 247) were removed and 3 separate normal saline emulsions prepared. These were inoculated intratesticularly into each of 3 normal rabbits. The popliteal nodes of rabbit No. 244, which had received 72 hours of streptomycin failed to cause syphilitic lesions or a positive Kahn reaction in a recipient rabbit in 141 days. At this time the rabbit died so that further study could not be carried out. The popliteal nodes of rabbit No. 247, which had received 96 hours of therapy likewise failed to produce clinical or serological evidence of syphilis in the recipient rabbit in 141 days. This rabbit subsequently developed testicular chancres, following inoculation with darkfield positive material. The rabbit inoculated with the popliteal glands of the control rabbit (No. 243) developed darkfield positive lesions in 41 days and a quantitative Kahn reaction of 800 units.

Comment. One report has already appeared regarding the use of streptomycin in 4 cases of human syphilis by Herrell and Nichols in collaboration with O'Leary.[3] Each patient received treatment for 10 days with a total dosage varying from 3.2 g to 10.0 g. Assuming that these patients were of average weight, approximately 60 kg, the dosage varied from 5.3 mg to 16.6 mg per kg per day. In the 3 cases with darkfield positive

[3] Herrell, W. E., and Nichols, D. R., *Proc. Staff Meet., The Mayo Clinic,* 1945, **20**, 449.

lesions, early improvement was noted in that the lesions became free of spirochetes. However, all 3 of these cases of syphilis later developed darkfield positive relapses.

The 2 rabbits here reported received approximately 3 to 5 times as much streptomycin on a daily kilogram basis as the above patients. In one rabbit there was apparent cure of the disease after 72 hours of treatment while in the other rabbit 96 hours of treatment failed to cure as demonstrated by testicular transfers. On the other hand, popliteal transfers of both rabbits performed 6 months after streptomycin therapy were negative. Employing the dosage schedule used in these 2 rabbits, the daily dose in a man weighing approximately 60 kg would be 2.5 g which does not represent excessive amounts of streptomycin. The drop in the quantitative Kahn reaction in the 2 rabbits which had received streptomycin is of significance when compared with the control. This same phenomenon was reported as having occurred in one of the patients of the Mayo group. It is important to note that the testicular emulsion of the rabbit which received 96 hours of treatment contained nonmotile spirochetes which took 120 days to cause syphilitic lesions in 2 recipient rabbits, whereas the 24- and 48-hour post treatment testicular transfers, as well as the control, required only 40 days.

In a recent report, Dunham and Rake[4] have likewise shown that streptomycin exerts an antisyphilitic effect in experimental syphilis in the rabbit. While their method of study differed entirely from that employed in these studies, the results are roughly comparable.

Summary. 1. Two syphilitic rabbits were treated with streptomycin for 72 hours and 96 hours respectively, with 42.5 mg per kg and 45.5 mg per kg per day respectively. 2. Darkfield examinations were positive at the end of 24 and 48 hours of treatment and transfers of testicular material from both rabbits caused syphilitic lesions in other rabbits in 40 days. 3. Darkfield examination was negative after 72 hours of treatment in the rabbit which had received 42.5 mg of streptomycin per kg per day. Transfer of one of this rabbit's testicles to 2 rabbits failed to cause syphilitic changes. 4. The rabbit which was treated for 96 hours with 45.5 mg of streptomycin per kg per day showed nonmotile spirochetes which were capable of causing syphilis in 2 rabbits. However, the lesions did not develop until 120 days after inoculation. 5. Popliteal transfers of the streptomycin-treated rabbits, performed 6 months after therapy, failed to cause syphilitic lesions in 141 days. The control's popliteal nodes caused changes in 41 days. 6. The quantitative Kahn reaction for syphilis showed a rapid drop in the rabbits which had received streptomycin. This eventuated in negativity whereas the control remained positive. 7. The results obtained in this preliminary experiment justify further investigation of the effects of streptomycin in experimental syphilis.

[4] Dunham, W. B., and Rake, G., *Science*, 1946, **103**, 365.

15389

Inositol, a Constituent of Thyroid Gland Effect on Perfused Rabbit's Heart.

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In connection with an investigation of physiologically active factors contained in thyroid substance, a commercial powder of desiccated thyroid gland (Wilson) of an iodine content of 0.55% was extracted with 95% ethyl alcohol in a Soxhlet apparatus. The extraction was so conducted that the alcohol in contact with the gland powder had a temperature of about 50° and was continued for at least 3 days. The brown alcoholic extract was left standing for 24 hours, after which time a solid substance was found at the bot-

tom of the container. This substance was dissolved in water; the insoluble part was filtered off and the clear solution was treated with norite and evaporated in a water bath to a small volume. Alcohol was added gradually until a precipitate began to be formed. While standing in the cold overnight, slightly yellow crystals were formed. By repeated crystallization from diluted alcohol, using the technic as described, colorless crystals were obtained. They had a corrected melting point of 223° and after drying at 110° gave the following analysis:

40.07 and 39.95% C; 7.04 and 7.10% H; 0.0% N. The substance showed no optical rotation and gave the Scherer reaction.

The theoretical values for inositol are 40% C and 6.67% H. A sample of commercial inositol was recrystallized and then gave a melting point of 223°. A mixture of the crystals from thyroid and inositol had the same melting point. The substance thus was identified as inactive inositol.

The quantity contained in the glandular material was not determined exactly, as the repeated crystallizations lead necessarily to losses. However, it is estimated that about 0.3% of free inositol was extracted from the thyroid powder.

The presence of inositol in aqueous extracts obtained from thyroids in a manufacturing process has been reported before by Tambach.[1] The quantity contained in the thyroid is suggestive of a special significance in the organism. Most of our attempts to find physiological effects of inositol which are not limited to the mere correction of a deficiency, have proved unsuccessful.

Brissemoret and Chevalier[2] have observed a toxic action of inositol on the rabbit's heart perfused by the Langendorff method. They added 0.05 and 0.1% of inositol to the perfusion fluid. In our experiments by the Langendorff method we replaced 10 and 20% of the 0.1% glucose in Locke's solution with inositol to avoid differences in ionic concentration that alone may cause a change in the rhythm of a perfused heart. The effect of both inositol products, the commercial and that isolated from thyroid, was identical in 6 different experiments and consisted in an immediate decrease in the auricular and ventricular amplitude followed by arrhythmias. Final death of the heart could be prevented by change to standard Locke solution as perfusion fluid, which effected prompt, but not quite complete, recovery. The concentrations of inositol of 0.01 and 0.02% are higher than occur physiologically for which reason no conclusion as to the significance of inositol in the function of the heart can be drawn.

Summary. Substantial quantities of optically inactive free inositol were extracted from dried thyroid glands. In the perfused rabbit's heart inositol causes a decrease in the amplitude and arrhythmias.

[1] Tambach, R., *Pharmazeutische Centralhalle*, 1896, **17**, 167.

[2] Brissemoret, A., and Chevalier, J., *C. R. Acad. Sciences*, 1908, **147**, 217.

15390 P

Absence of Appreciable *L. casei* Factor Effect in Anti-Pernicious Anemia Liver Extract.

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The absence of considerable quantities of "free" *L. casei* factor from concentrated anti-pernicious-anemia liver extracts[1] is in contrast with the effect of the "liver" form of *L. casei* factor in producing remission of pernicious anemia.[2-4] The possibility remained that "free" *L. casei* factor could be liberated

[1] Clark, G. W., *Am. J. Med. Sci.*, 1945, **209**, 520.

[2] Vilter, C. F., Spies, T. D., and Koch, M. B., *South. Med. J.*, 1945, **38**, 781; Moore, C. V., Bierbaum, O. S., Welch, A. D., and Wright, L. D., *J. Lab. and Clin. Med.*, 1945, **30**, 1056.

in vitro or *in vivo* from the maturation factor which is present in such liver extracts.

In the present investigation, concentrated parenteral liver extract, 15 U.S.P. units per cc, was incubated with dried chicken pancreas, which contained "vitamin B_c conjugase." The content of "free" *L. casei* factor after incubation was found to be 1.0 μg per cc by *L. casei* assay and 0.8 μg per cc by *S. lactis R* assay, against synthetic "liver" *L. casei* factor standard.

The effect of various samples of the same type of liver extract on chicks deficient in *L. casei* factor was then measured. Day-old New Hampshire Red chicks were placed on the diets. Ten chicks were used in each group. The basal diet had the following composition: glucose (cerelose) 58.5 g, purified casein (Labco) 20 g, gelatin 8 g, calcium gluconate 5 g, cystine 0.4 g, choline chloride 0.2 g, inositol 0.1 g, bone ash 2 g, NaCl 0.6 g, KH_2PO_4 0.45 g, K_2HPO_4 0.6 g, $MgSO_4$ 0.25 g, $MnSO_4,4H_2O$ 0.05 g, ferric citrate 0.05 g, $CuSO_4,5H_2O$ 2 mg, $Al_2(SO_4)_3,18H_2O$ 1.6 mg, zinc acetate 1.4 mg, KI 0.6 mg, cobalt chloride 0.4 mg, nickel chloride 0.2 mg, calcium pantothenate 5 mg, niacinamide 5 mg, riboflavin 1 mg, pyridoxine HCl 1 mg, thiamine HCl 1 mg, p-aminobenzoic acid 1 mg, 1-acetoxy-2 methyl-4 naphthyl sodium phosphate 0.5 mg, (dl) biotin .04 mg* to which were added vitamin A 1500 U.S.P. units, vitamin D 200 A.O.A.C. units, mixed tocopherols 34 mg, dissolved in corn oil (Mazola) to a total of 3 g. Table I illustrates the results of a typical experiment in which concentrated parenteral liver extract was injected into chicks at a rate which corresponded to approximately 0.4 U.S.P. unit of maturation factor per day. This level of dosage had no effect on the chicks under conditions in which a marked growth response was obtained with 0.5 or 1.0 mg of "liver" *L. casei* factor added per kilo of diet, which resulted in a daily intake of 5 to 20 μg of added *L. casei* factor.

TABLE I.

Effect of Various Supplements on Growth of Chicks on Diet Deficient in *L. casei* Factor.

Supplement, or other treatment	Gain in g	
	In 3 wk	In 4 wk
None	47	98†
0.1 cc liver extr., 15 units per cc injected twice weekly	48	66‡
1.0 mg synthetic "liver" *L. casei* factor added per kilo of diet*	113	222

* Other experiments showed that the growth response with 0.5 mg was as great as with 1.0 mg.
† 6 survivors.
‡ 4 "

It has been shown elsewhere[5] that chicks under similar conditions respond markedly to daily injections of 5 to 10 μg of "liver" *L. casei* factor.

Results with pernicious anemia patients[3,4] indicate that an effect corresponding approximately to 1 U.S.P. unit of maturation factor in injectable liver extract is obtained by injecting or feeding 1 to 10 mg of "liver" *L. casei* factor daily, while a level of 0.35 mg produced an incomplete response.[4] From this it would seem that 0.4 U.S.P. unit of maturation factor would be equivalent to at least 400 μg of "liver" *L. casei* factor in the treatment of pernicious anemia. The present investigation indicates that *L. casei* factor was not liberated in appreciable quantities from concentrated liver extract by treatment with vitamin B_c conjugase as supplied by dried chicken pancreas. The maturation factor as present in liver extract was ineffective for chick growth when injected at a level which would correspond, on an anti-pernicious-anemia basis, to an estimated dosage of an equivalence of 400 or more μg of "liver" *L. casei* factor daily; moreover, chicks under these conditions showed a marked growth response to a daily intake of 5 to 10 μg of "liver" *L. casei* factor.†

[3] Doan, C. A., Wilson, H. E., Jr., and Wright, Claude-Starr, *Ohio State Medical J.*, 1946, **42**, 139; Spies, T. D., *J. A. M. A.*, 1946, **130**, 474.

[4] Welch, A. D., Heinle, R. W., and Moore, C. V., paper presented at Atlantic City meeting, American Chemical Society, April, 1946.

* Kindly provided by Dr. B. R. Baker.

[5] Frost, D. B., Dann, F. P., and McIntire, F. C., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 65.

† Unpublished results by Daft and co-workers (personal communication from Dr. F. S. Daft) indicate that concentrated parenteral liver extract, 15 U.S.P. units per cc, was found to be ineffective against a deficiency of *L. casei* factor as produced in rats by feeding a purified diet to which a sulfonamide had been added.

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Vagotomy Fails to Protect Against Histamine-Provoked Ulcer.*

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The occurrence of chronic peptic ulcer following bilateral vagotomy in rabbits has been observed by a number of investigators.[1] Recently, supradiaphragmatic vagus section has been used in the surgical treatment of gastroduodenal ulcer,[2] on the thesis that, the hypersecretion of night gastric juice common in ulcer patients is very largely neurogenic in origin. Gastrojejunostomy was used in some cases in addition to vagus section because of high grade pyloric stenosis. It is the purpose of this report to indicate our observations regarding vagus section in a few of the common laboratory animals (dog, cat and rabbit) and to note whether bilateral vagotomy or gastrojejunostomy and bilateral vagotomy will protect against the histamine-in-beeswax provoked ulcer.

Method. These experiments were carried out on 33 animals consisting of 13 dogs, 6 cats and 14 rabbits. Bilateral vagotomy was performed on these animals. After an average interval of 9 days in the dogs, 7 days in the cats and 8 days in the rabbits, the administration of the histamine-in-beeswax mixture prepared after the method of Code and Varco[3] was begun. Thirty milligrams of this mixture was injected intramuscularly each evening in the dog and rabbit, while 15 mg was injected in the cat. Following the injection the dogs' feed pans were removed and no more food was given until the following morning. Unless the animals succumbed from either aspiration pneumonia which often occurred after vagus section, or from ulcer provoked by the histamine implantation, they were sacrificed after varying periods of time to see if any ulcer were present at that time.

Operative Procedure. Supradiaphragmatic Section. This procedure was used on all the dogs (13), 2 cats and 9 rabbits. A long intercostal incision was made either in the fifth or sixth interspace. The muscles were then divided and retracted. Under positive pressure cyclopropane-oxygen anesthesia, the pleura was opened. The lungs immediately collapsed and controlled respiration was used during the operation. The left lung was retracted out of the field and the esophagus in the mediastinum became visible. The pleura over the lower esophagus was then opened and the organ mobilized. The left vagus and all visible and palpable fibers were then tied and sectioned accompanied by removal of a segment of 1 to 2 cm of the nerve. This procedure was repeated on the right vagus. In addition, in the dog, the proximal ends were sutured to the pleura lateral to the esophagus, as previously described by Dragstedt and Schafer.[2] This maneuver was not technically feasible in the rabbit and cat. The chest was then closed with interrupted cotton sutures.

Infradiaphragmatic Section. This procedure was used on 4 cats and 5 rabbits. These animals have a rather long infradiaphragmatic portion of esophagus and the vagi may be identified readily within the abdomen. Using a midline incision and pulling the stomach out on the abdominal wall and then downward and to the right, the infradiaphragmatic esophagus can be brought readily into view. The visible and palpable left and right vagi were then tied and sectioned and ½ to 1 cm of nerve removed. In addition, a circular area

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† National Cancer Trainee.

1 Beozell, J. M., and Ivy, A. C., *Arch. Path.*, 1936, **23**, 213.

2 Dragstedt, L. R., and Schafer, P. W., *Surgery*, 1945, **17**, 742.

3 Code, C. F., and Varco, R. L., Proc. Soc. Exp. Biol. and Med., 1940, **44**, 475.

TABLE I.
Production of Ulcer in Dogs Following Supradiaphragmatic Vagotomy (30 mg Histamine-in-Beeswax Mixture Daily).

Dog No.	Wt in lb	No. of injections	Results	Remarks	Wt of stomach, g
419	38	45	Antral ulcer; marked duodenitis; erosions in fundus	Sacrificed after 45 days; stomach dilated and atonic appearing	129
420	40	45	Marked duodenitis, erosions in antrum	Sacrificed after 45 days; stomach dilated and atonic appearing	160
422	26	21	Normal stomach and duodenum	Sacrificed after 45 days	145
450	28	32	Perforated ulcer lower esophagus	Died of perforation of esophageal ulcer into left thorax, stomach dilated	95
453	36	40	Erosions in antrum and duodenum	Sacrificed after 40 days, markedly dilated stomach	163
454	28	12	3 duodenal ulcers, antral erosions	Died in 12 days, pneumonia probably caused by aspirated bile and gastric juice, bile present in bronchi	155
457	26	8	4 duodenal ulcers, (2 perforating)	Pneumonia, dog vomiting, bile in bronchi	138
458	30	40	Normal stomach and duodenum	Tremendously dilated stomach	185
459	36	14	3 small duodenal ulcers	Dead 2 weeks, empyema and pneumonia	120

Number of dogs injected: 9.
Number of dogs with ulcer and/or erosion: 7.

TABLE II.
Production of Ulcer in Dogs Following Supradiaphragmatic Vagotomy and Gastrojejunostomy (30 mg Histamine-in-Beeswax Mixture Daily).

Dog No.	Wt in lb	No. of injections	Results	Remarks	Wt of stomach, g
428	31	4	Normal stomach, duodenum and jejunum	Died of obstruction due to jejuno-jejunal intussusception	165
430	41	17	Perforated jejunal ulcer	Died of peritonitis	140
434	39	45	Large penetrating jejunal ulcer	Sacrificed 45 days, base of ulcer formed by adhesions and loop of bowel	130
435	45	45	Jejunitis, normal	Sacrificed 45 days, large atonic stomach	136

No. of dogs injected: 4. No. of dogs with ulcer and/or erosion: 2.

TABLE III.
Production of Ulcer in Cat with Bilateral Vagotomy and Histamine-in-Beeswax (15 mg of Histamine-in-Beeswax Given Intramuscularly Once Daily).

Cat No.	Wt in kg	No. of injections	Type of vagotomy	Results	Remarks	Wt of stomach, g
1	2.5	10	Infradiaphragmatic	Antral erosions	Sacrificed after last injection	26.2
2	3	21	”	Esophageal ulcers Antral ulcer	Severe vomiting during last few days	19.2
3	2	8	Supradiaphragmatic	Duodenal ulcer (perforated)	Received injections every other day	24.3
4	3	21	Infradiaphragmatic	2 duodenal ulcers (1 perforated)	Sacrificed after 22 days	24.5
5	1.5	31	”	No ulcers	Sacrificed in 32 days	16.1
6	2.5	31	Supradiaphragmatic	3 large duodenal ulcers	”	20.5

No. of cats injected: 6.
No. of cats with ulcer and/or erosion: 5.

TABLE IV.
Production of Ulcer in Rabbit with Bilateral Vagotomy and Histamine-in-Beeswax (30 mg of Histamine-in-Beeswax Given Intramuscularly Once Daily).

Rabbit No.	Size	No. of injections	Type of vagotomy	Results	Remarks	Wt of stomach, g
4	Adult	7	Infradiaphragmatic	Duodenal ulcer and bleeding antral ulcer	Sacrificed 8 days postoperatively	26.5
5	,,	21	,,	Bleeding duodenal ulcer	Empyema due to perforation of fundus ulcer into thorax	24.2
9	,,	10	Supradiaphragmatic	Duodenal ulcer	Sacrificed in 11 days	23.8
10	,,	36	,,	No ulcer	Sacrificed in 37 days	23.2
11	,,	36	,,	,,	,,	21.2
12	,,	48	,,	,,	Sacrificed in 48 days	24.3
13	,,	2	,,	Perforated duodenal ulcer	Postmortem 5 minutes after convulsion, blood in intestinal tract	19.2
14*	,,	13	Infradiaphragmatic	2 esophageal ulcers	Sacrificed in 14 days	23.5

* This rabbit received 15 mg of histamine-in-beeswax mixture for 8 days and 22½ mg for 5 days.
No. of rabbits injected: 8.
No. of rabbits with ulcer and/or erosion: 5.

TABLE V.
Production of Ulcer in Rabbit with Bilateral Vagotomy. (No Histamine).

Rabbit No.	Size	Type of vagotomy	Results	Remarks	Wt of stomach, g
1	Adult	Supradiaphragmatic	Perforating fundus ulcer	Severe pylorospasm, sacrificed 5 days postoperatively	20.1
2	,,	,,	No ulcers	Sacrificed 29 days postoperatively	21.7
3	,,	,,	,,		
6	,,	,,	Large antral ulcer	Same 29 days	22.2
				Same 36 days	28
7	,,	Infradiaphragmatic	Bleeding cardiac erosions	Same 36 days, very edematous stomach	32
8	,,	,,	No ulcers	Sacrificed 13 days postoperatively	21.5

on the wall of the esophagus was then denuded of peritoneum to insure destruction of all small vagus branches. The incision was then closed.

Gastrojejunostomy. The dogs were anesthetized with intravenous nembutal 15 mg per pound. Anesthesia was continued with intratracheal ether. Continuous gastric suction was applied during and immediately after surgery. Under aseptic conditions, a midline incision was made. The duodenum was transected and inverted immediately distal to the pyloric sphincter. One or 2 cm of stomach antrum including the pyloric sphincter was resected. Continuity of the intestinal tract was completed by aseptic gastrojejunostomy just beyond the ligament of Treitz.

Postoperatively, the dogs were fed parenterally for 72 hours with 10% glucose in saline solution containing some isotonic gelatine in normal saline. After 72 hours, water was allowed and the diet gradually increased thereafter until in one week the regular diet of tablescraps, horsemeat and kibbles was tolerated. This was reinforced with vitamins A, B, C and D. After an average interval of 92 days bilateral supradiaphragmatic vagotomy as described above was performed.

Experiments. Dogs. Thirteen dogs were used in this series. Bilateral supradiaphragmatic vagotomy was performed on these animals, 4 animals having had gastrojejunostomy performed previously. After a sufficient recovery period, the daily administration of the histamine-in-beeswax mixture was begun. One animal (dog 422) was sacrificed after 21 days as a test dog.

Cats. Six cats were used and all were sub-

Fig. 1.

Stomachs of dogs with bilateral supradiaphragmatic vagotomy and gastrojejunostomy receiving histamine-in-beeswax. A. (Top) Dog 434 (left), large jejunal ulcer. B. (Bottom) Dog 430, (right), perforation of jejunum, died 17 days after commencement of histamine injections of peritonitis.

jected to bilateral vagus section and histamine-in-beeswax implantation. In addition, 3 control rabbits were subjected to a daily implantation of 30 mg histamine-in-beeswax mixture for 28 days.

Rabbits. Fourteen rabbits were used in this series. Six of these had bilateral vagotomy only, while 8 had both bilateral vagotomy and received histamine-in-beeswax in addition.

Results. The incidence of histamine-in-beeswax provoked ulcer or erosion (gastric and/or duodenal) following bilateral vagotomy and combined vagotomy and gastrojejunostomy procedure is shown in Tables I, II, III and IV. The occurrence of ulcer or erosion in the rabbit with bilateral vagus section alone is shown in Table V. In none of the control rabbits was ulcer or erosion observed.

Comment. It is worthwhile to comment on some of the postoperative complications attendant on bilateral vagus section. Marked atony and dilatation of the stomach was observed in most animals. The stomachs were not thickened on section but grossly they were very large. The presence of esophageal ulcer following histamine implantation was not an uncommon finding. Repeated vomiting was frequent in the dog and difficulty in swallowing was observed in many. This occurrence may explain the finding of esophageal ulcers and also the presence of bile in the bronchi of some of the dogs (Table I).

Discussion. These results indicate that bilateral vagus section or gastrojejunostomy and vagotomy do not protect against ulcer or erosion (gastric and/or duodenal) produced by chronic histamine stimulation. In addition, bilateral vagus section alone in the rabbit may produce ulcers. It has been suggested that this phenomenon may be due to trophic disturbances in the gastric wall brought about by vagotomy.[1] It has also been suggested that vagus fibers antagonize the vasoconstrictor action of the splanchnic nerves and when sectioned, the vasoconstrictor influence is unopposed.[4] It should be mentioned, however, that not only vagotomy, but also splanchnicectomy may be followed by gastric

[4] Alvarez, W. S., Hosoi, K., Overgard, A., and Ascanco, H., *Am. J. Physiol.*, 1929, **90**, 631.

ulcer in rabbits.[4] Recent evidence from this laboratory has shown that both vasoconstriction[5] and venous stasis[6] may aid and abet the ulcer diathesis by producing anoxic areas which then fail to resist the digestive action of the peptic mixture. Inasmuch as the effect of histamine is directly on the parietal cell, in the gastric tubule, the results of these experiments are not to be interpreted as a criticism of the application of vagotomy to the problem of ulcer in man.

Conclusions. 1. Bilateral vagotomy (infra- and supradiaphragmatic) failed to protect against ulcer or erosion (gastric and/or duodenal) produced by chronic histamine action in the dog, cat and rabbit.

5 Baronofsky, I., and Wangensteen, O. H., *Bull. Am. Coll. Surg.*, 1945, **30**, 59.

6 Baronofsky, I., and Wangensteen, O. H., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 234.

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Hemagglutination by Amniotic Fluid from Normal Embryonated Hen's Eggs.*

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During studies on the detection of influenza virus in throat washings[1] an agglutinin for guinea pig erythrocytes was noted in the amniotic fluid from certain inoculated eggs. Chicken red cells were not agglutinated by these fluids. This suggested that the fluids might contain the "O" form of influenza virus described by Burnet,[2] which grows only in the amniotic sac and which agglutinates guinea pig cells to higher titers than chicken cells. Subsequent study of amniotic fluid from uninoculated eggs, however, revealed that normal fluid also contained an agglutinin for guinea pig erythrocytes but not for chicken red cells. The phenomenon was unrelated to and independent of the presence of influenza virus.

The agglutinin was capable of clumping the erythrocytes of a wide variety of animals. It was present in the egg white of non-embryonated eggs and the albumin sac of embryonated eggs, gaining access to the amniotic sac after the establishment of the communication between it and the albumin sac which normally takes place between the 11th and 13th days of incubation.[3] It was associated with the globulin fraction of the egg white, was thermolabile, and was adsorbed to erythrocytes during agglutination. The characteristics of the hemagglutinin are described in this report.

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1 Commission on Acute Respiratory Diseases, to be published.

2 Burnet, F. M., and Bull, D. R., *Australian J. Exp. Biol. and Med. Sc.*, 1943, **21**, 55.

3 Hirota, J., *J. Coll. Sc., Imp. Univ. Japan*, 1894, **6**, 339. Cited in Needham, J., *Chemical Embryology*, London, Cambridge University Press, 1931, vol. 2, p. 1100.

TABLE I.
Agglutination of Erythrocytes of Different Species by Amniotic Fluid from Normal 14- to 17-Day Embryos, Normal Adult Chicken Serum, and Normal 17-Day Embyro Serum.

Erythrocytes	Final agglutinative titer		
	Amniotic fluid pool	Adult chicken serum pool	Chick embryo serum pool
Mouse (Swiss)	32	16	<2
Rabbit	32		
Dog	16	32 or >	<2
Guinea pig	8	32 or >	<2
Cotton rat	8		<2
Horse	8	16	<2
Human Group O	8		
" " A	4		
" " B	4		
" " AB	2		
Chimpanzee	4		
Hamster (Syrian)	4		
Cat	4	8	<2
Mule	4	<2*	<2
Pigeon	4	32 or >	<2
Monkey (Rhesus)	2		
Ferret	<2*		
Sheep	<2*	8	<2
Cow	<2*	2	<2
Toad	<2*	8	<2
Chicken	<2	<2	<2

* Agglutination doubtful in 1:2 dilution.

Materials and Methods. Source of materials. The eggs from which the hemagglutinin was derived all came from New Hampshire Red chickens. Fertile eggs were incubated at 37.7°C (100°F). Egg white, albumin sac contents, allantoic fluid, and amniotic fluid were obtained using sterile technic and were stored at 4°C without preservative. Pools of amniotic fluid were made up of individually tested fluids known to agglutinate guinea pig cells.

Preparation of erythrocytes. Erythrocytes were obtained from citrated blood. They were washed 3 times in buffered saline[4] and packed for 15 minutes at 1500 R.P.M. in the horizontal centrifuge. A 1.5% suspension by volume of packed cells in buffered saline was used throughout except as indicated in the text. Erythrocytes were used within 24 hours of the time they were obtained.

Titration of hemagglutinin. All dilutions were made in buffered saline. To 0.1 ml of the test fluid in an 11 x 75 mm tube was added 0.1 ml of cell suspension. A control included in each test consisted of 0.1 ml of buffered saline mixed with 0.1 ml of cell suspension. After thorough shaking the mixtures were incubated at 4-10°C except as indicated. They were observed at frequent intervals for agglutination. The reciprocal of the highest dilution in which agglutination was observed was recorded as the titer, which was expressed in terms of final dilution.

Experiments and Results. Description of the agglutination. The agglutination was first noted in amniotic fluids from 16-day embryos. Clumping was difficult to discern while the cells were settling, but after complete sedimentation had taken place the cells formed a characteristic pattern on the bottom of the tube. They clung to the entire curve of the bottom, covering it in a uniform, finely granular sheet. Later they slid downward in irregular fashion from the upper and outer edges, the resulting pattern having a stellate configuration with its edges accentuated by the constant accession of cell masses. Eventually the pattern became identical with that of the saline control, in which the cells settled in a homogeneous, compact, circular disc having a regular, sharply demarcated periphery.

This pattern closely resembles that due to

[4] Eagle, H., *The Diagnosis of Syphilis*, St. Louis, C. V. Mosby Co., 1938.

TABLE II.
Distribution of Hemagglutinin for Erythrocytes of Several Species in Normal Embryonated Hen's Eggs by Day of Development.

Day of development of embryo	Amniotic fluid			Allantoic fluid	
	Mouse cells	Guinea pig cells	Chicken cells	Guinea pig cells	Chicken cells
9	N.T.*	0/12	0/12	N.T.	N.T.
11	0/9	0/48	0/48	0/36	0/36
12	3/8	0/48	0/36	0/36	0/36
13	8/8	2/47	0/35	0/24	0/24
14	N.T.	12/41	0/35	0/33	0/33
15	N.T.	19/43	0/36	0/36	0/36
16	10/11	16/44	0/36	0/36	0/36
17	N.T.	23/36	0/36	0/34	0/34

* N.T. = not tested.
Numerator = No. of positive fluids.
Denominator = No. of fluids tested.

influenza viruses A and B,[5,6] vaccinia,[7] and pneumonia virus of mice[8] when they are diluted almost to their agglutinative endpoints, and is indistinguishable from that caused by metallic ions such as Ca++ and Mg++.[9,10] Agglutination caused by these agents and by normal amniotic fluid likewise disappears when the tube is gently shaken and reappears when the cells settle again.

The type of agglutination was the same irrespective of the source of the hemagglutinin, but since there was a considerable variation in viscosity of the fluids tested, the erythrocyte sedimentation rate varied accordingly. For this reason no time interval for reading agglutination was set; instead the settling process was observed frequently until complete. If the cells covered the bottom in a uniform sheet and later assumed a stellate pattern the amniotic fluid was considered to contain hemagglutinin. Patterns corresponding to those of the saline controls were considered negative. Patterns which deviated in any particular from either of the 2 mentioned above were considered doubtful.

5 Hirst, G. K., *Science*, 1941, **94**, 22.

6 McClelland, L., and Hare, R., *Canad. Pub. Health J.*, 1941, **32**, 530.

7 Nagler, F. P. O., *Med. J. Australia*, 1942, **1**, 281.

8 Mills, K. C., and Dochez, A. R., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 140.

9 Northrup, J. H., and Freund, J., *J. Gen. Physiol.*, 1924, **6**, 603.

10 Landsteiner, K., *The Specificity of Serological Reactions*, Cambridge, Harvard University Press, 1945.

Agglutinability of erythrocytes from different species. A survey of the agglutinability of the erythrocytes of 18 different species was made in order to determine whether a systematic variation in this regard existed. A single positive pool of normal amniotic fluid was used for the experiment. The cells were also tested with normal adult and 17-day embryo chicken serum.

No systematic variation in agglutinability was apparent (Table I). Mouse and rabbit cells gave the highest agglutinative titer. No apparent relation existed between the agglutinative titer of normal amniotic fluid and that of normal adult chicken serum for erythrocytes of the respective species examined. The red cells of none of the species tested agglutinated in serum from normal 17-day chick embryos.

Effect of temperature of incubation on agglutinative titer. The titer of the hemagglutinin depended on the temperature at which the agglutination reaction was carried out. It was considerably greater at low than at higher temperatures. For example, in a representative experiment the titer of an amniotic fluid pool for mouse erythrocytes was 32 when the reaction was carried out at 8°C, 8 at 30°C, 2 at 37°C, and <2 at 56°C. As has been indicated, incubation of agglutination tests was routinely carried out at 4-10°C.

Relationship between the appearance of hemagglutinin and embryonic development. In order to determine whether the age of the embryos was correlated with the appearance

TABLE III.
Approximate Agglutinin Titers for Mouse and Rabbit Erythrocytes of Fractions of Pooled Egg White and of Pooled Albumin Sacs from 12-Day Embryos.

Fraction		Titer* according to source of hemagglutinin			
		Egg white		Albumin sac	
% $(NH_4)_2SO_4$ saturation	Precipitate or supernatant fluid	Mouse erythrocytes	Rabbit erythrocytes	Mouse erythrocytes	Rabbit erythrocytes
No fractionation		64	64	96	96
29	ppt.†	6	6	8	8
	super.	16	16	48	48
48	ppt.	64	32	32	32
	super.	<8	<8	<12	<12
100	ppt.	<4	<4	<6	<6
	super.	<8	<8	<12	<12

* Final titer (by weight of original specimen).
† ppt. = precipitate; super. = supernatant fluid.

of the hemagglutinin in the amniotic fluid, and also whether it appeared at any time in the allantoic fluid, embryo fluids were examined daily with mouse, guinea pig, and chicken erythrocytes. Amniotic fluids from embryos incubated less than 12 days failed to cause agglutination (Table II). The agglutinin for mouse cells was first detected on the 12th day, and for guinea pig cells on the 13th day, and was found in the majority of eggs from then on. Chicken cells failed to agglutinate in any fluid.

There was a definite correlation between the viscosity of the amniotic fluid and the presence of hemagglutinin. Thin, watery fluids contained none; thick, viscous fluids usually agglutinated the red cells. This correlation may be considered simply another manifestation of that existing with the degree of embryonic development, since the amniotic fluid becomes thickened when the contents of the albumin sac find their way into it.[3]

No hemagglutinin for any of the 3 species of erythrocytes was detected in the allantoic fluid of embryos of any age, regardless of whether the amniotic fluid contained hemagglutinin.

Nature of the hemagglutinin. The appearance of hemagglutinin in the amniotic but not the allantoic fluid at the approximate time of rupture of the content of the albumin sac into the amnion, and the correlation between increased viscosity of the amniotic fluid and hemagglutinative properties, suggested that the content of the albumin sac was the source of the agglutinin. The albumin sac contains inspissated egg white; therefore, both the white of non-embryonated eggs, and albumin sac contents of 10 11- to 12-day embryos, were examined for the hemagglutinin. It proved to be present in all specimens tested. Thorough mixing of the specimens was essential.

To determine which fraction contained the agglutinin, ammonium sulfate, first in half saturation, then in full saturation, was added to amniotic fluid having a titer of 16 for mouse erythrocytes. The precipitate resultant from half saturation (globulins), when dissolved in 0.85% sodium chloride solution, had a titer of 12, while that resultant from subsequent full saturation (albumins) had a titer of <2.

The experiment was repeated with egg white and albumin sac contents, using successively 29, 48, and 100% saturation with ammonium sulfate. Samples of the respective supernatant fluids were dialyzed at 4°C for 2 days against distilled water, then for a few hours against 0.85% sodium chloride solution. The latter was again used as a solvent for the precipitates.

The results indicated that most of the hemagglutinin was associated with the fraction precipitated by 48% ammonium sulfate ("pseudoglobulins") (Table III). To verify the fact that the albumin fraction contained no hemagglutinin, a sample of purified oval-

TABLE IV.
Inactivation by Heat of Fresh Egg White Agglutinin for Mouse Erythrocytes, According to Temperature and Time of Exposure.

Temperature (C)	Titer* according to time of exposure					
	5 min	10 min	30 min	60 min	4 hr	20 hr
37	32	32	32	32	32	32
48	32	32	32	32	16	<4
56	32	32	32	16	8	<4
60†	8	8	4	4	—	—
65†	8	4	<4	<4	—	—

* Titer of the egg white for mouse erythrocytes before heating = 32.
† The egg white solution began to coagulate within 5 minutes at 60 and 65°C.

bumin‡ was tested with mouse, rabbit, and guinea pig erythrocytes. Agglutination did not take place.

Thermolability of the hemagglutinin. As would be expected in view of the properties described above, the hemagglutinin was inactivated by heat. Fresh egg white having an agglutinative titer of 32 for mouse erythrocytes was heated for various periods at different temperatures (Table IV). It was completely inactivated at 48°C in 20 hours. At higher temperatures inactivation proceeded more rapidly. Exposure to 37°C for 20 hours, however, did not decrease the titer.

Adsorption of agglutinin to erythrocytes. To determine whether the agglutinin was adsorbed to erythrocytes, packed mouse red blood cells were added to a positive amniotic fluid pool to make a 2% suspension by volume. The mixture was incubated at 4°C and aliquots were drawn off at intervals after mixing, following which the cells were immediately separated by centrifugalization and the supernatant fluids set aside. The supernatant fluids were then tested simultaneously with mouse erythrocytes to determine the titer of residual hemagglutinin. The titer decreased from 32 to 4 within 30 seconds and was <4 within 10 minutes, indicating adsorption had occurred. Elution did not take place within 24 hours.

The same experiment was done with chicken erythrocytes and the same amniotic fluid pool. The titer remained at 32 for the duration of the experiment, showing that the chicken cells did not adsorb the agglutinin.

To determine whether the agglutinin eluted at 37°C, the same amniotic pool was adsorbed to mouse erythrocytes at 4°C for 10 minutes. The supernatant fluid was removed and found to have a titer of <4. An equal volume of 0.85% sodium chloride solution was added to the erythrocytes and the suspension incubated at 37°C. Elution did not occur within 6 hours.

Discussion. The agglutination of red blood cells by egg white and extraembryonic fluids containing egg white, is not a unique phenomenon. In addition to specific antisera and the hemagglutinins which are present in certain normal sera, numerous other agents which can cause hemagglutination have been described. Ricin, abrin, and crotin, and other substances derived from plants, have this ability.[10] Influenza viruses A and B,[5,6] vaccinia,[7] and the viruses of Newcastle disease,[11] fowl plague,[12] mouse pneumonitis,[8] and mumps[13] agglutinate red cells. Certain inorganic colloidal acids or bases, such as colloidal silicic acid, and basic proteins such as protamines can induce hemagglutination.[10] Numerous metallic ions[9,10] have this property. These hemagglutinating agents, in common with the hemagglutinin in egg white, albumin sac content, and normal amniotic fluid, are selective in their action, failing to agglutinate the

‡ A sample of purified ovalbumin, shown to be homogeneous by means of electrophoretic determinations carried out by Dr. G. R. Cooper, was kindly furnished by Dr. A. R. Taylor, of the Department of Experimental Surgery, Duke University, School of Medicine.

11 Burnet, F. M., *Australian J. Exp. Biol. and Med. Sc.*, 1942, **20**, 81.

12 Lush, D., *J. Comp. Path. and Therap.*, 1943, **53**, 157.

13 Levens, J. H., and Enders, J. F., *Science*, 1945, **102**, 117.

erythrocytes of certain species, and agglutinating those of others at different titers.

The resemblance in some respects of the behavior of normal amniotic fluid hemagglutinin to that of influenza virus is striking and assumes practical importance because of the value of the amniotic route of inoculation for the primary isolation of influenza virus.[1,14,15] Embryos of that degree of development in which the probability of finding normal amniotic fluid hemagglutinin is greatest are best for the isolation of influenza virus. Burnet[2] has described a form of influenza virus, the "O" form, which grows well only in the amniotic sac and which agglutinates guinea pig cells better than chicken cells. It is readily apparent that the normal amniotic fluid agglutinin, which agglutinates guinea pig cells but not chicken cells, could be mistaken for the "O" form of influenza virus if the identification were allowed to depend on the demonstration of hemagglutination in undiluted fluid and were not verified by other procedures. The phenomenon described by Burnet, however, was not due to normal amniotic fluid agglutinin. The titer of "O" influenza virus was usually higher than that of normal amniotic fluid agglutinin; in most instances there was concurrent agglutination of both guinea pig and chicken cells by "O" strains; and the strains were identified as influenza virus by serological methods. Attention should also be called to the fact that human erythrocytes of all blood groups are agglutinated by the normal amniotic fluid factor. Human erythrocytes have been routinely used for the agglutination reaction with influenza virus in some instances.[16]

It appears that the hemagglutinin, which is associated with the globulin fraction of egg white, gains access to the amniotic fluid after the rupture of the albumin sac into the amniotic sac. This accounts for the fact that amniotic fluid is free from hemagglutinin until the 12th day of development, and that those fluids containing the hemagglutinin are of increased viscosity. The thermolability of the agglutinin is consonant with the fact that it is associated with the globulin fraction. The agglutinin is adsorbed to mouse erythrocytes, which are agglutinated by it, but is not adsorbed to chicken erythrocytes, which are not agglutinated by it.

The agglutination of certain strains of *Staphylococcus aureus* by allantoic fluid from normal 10-day embryonated hen's eggs has been reported by Shrigley.[17,18] In the earlier report[17] he mentions that a limited number of observations suggest that amniotic fluid may also cause the reaction, but that albumin does not. This phenomenon therefore does not appear to bear any relationship to the hemagglutination reaction.

Summary. A hemagglutinin appears in the amniotic fluid of normal chick embryos between the 11th and 13th days of incubation. The erythrocytes of 13 of 18 species tested are agglutinated. Mouse and rabbit cells are agglutinated to the highest titer. Guinea pig cells are also agglutinated, but chicken cells are not. Hemagglutination occurs best in the cold.

The hemagglutinin is associated with the globulin fraction of egg white. It gains access to the amniotic fluid when the content of the albumin sac ruptures into the amniotic sac between the 11th and 13th days of development of the embryo.

The hemagglutinin is thermolabile. It is adsorbed by mouse but not by chicken erythrocytes.

14 Burnet, F. M., *Australian J. Exp. Biol. and Med. Sc.*, 1940, **18**, 353.

15 Hirst, G. K., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 155.

16 Personnel of Naval Laboratory Research Unit No. 1, *U. S. Naval Med. Bull.*, 1943, **41**, 114.

17 Shrigley, E. W., *Science*, 1945, **102**, 64.

18 Shrigley, E. W., *Yale J. Biol. and Med.*, 1945, **18**, 81.

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Effect of Penicillin and Streptomycin on Bacterial Contamination of Chick Embryos Inoculated with Unfiltered Sputums.

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(Introduced by A. R. Dochez).

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In a previous communication the use of penicillin for controlling bacterial contamination in chick embryos inoculated with unfiltered secretions of the respiratory tract was described.[1] It was noted, however, that while this method had been successfully employed for the isolation of influenza and herpes viruses, embryos not infrequently died as the result of infection with bacteria which were resistant to the action of penicillin; in the main these microorganisms were Gram-negative bacilli of the Proteus or Pseudomonas groups, or coliforms that were not further identified. Since streptomycin has been shown to exercise a potent bacteriostatic and bactericidal effect on many Gram-negative bacteria[2,3] it therefore seemed of interest to determine whether this compound, either singly or in combination with penicillin, was superior to penicillin alone in decreasing bacterial contamination and promoting the survival rate of chick embryos inoculated with unfiltered sputums.

Experimental. Specimens of sputum were obtained from 60 patients suffering from various infections of the respiratory tract including bacterial pneumonia, primary atypical pneumonia, bronchiectasis, acute tracheobronchitis and the common cold. In many instances specimens were deliberately collected from patients whose antecedent sputum cultures had revealed the presence of Gram-negative bacilli.

The sputums were freshly collected and either ground in a sterile mortar with quartz sand or emulsified in a Waring blendor with an equal volume of sterile physiological saline, followed by centrifugation in a Swedish angle centrifuge at 3000 R.P.M. for 10 minutes. The supernatant fluid of each specimen was then inoculated in 0.3 ml amounts into 8 developing hen's eggs which had been incubated for 10 or 11 days at 39°C. Two of the eggs were reserved as controls, 2 received 500 units of penicillin in a volume of 0.1 ml, 2 received 500 units of streptomycin in a similar volume, and 2 received both penicillin and streptomycin. All inoculations were made into the allantoic cavity.

After inoculation the eggs were incubated at 35°C and were candled daily. Eggs in which the embryos appeared to have died were opened at once, Gram-stained smears of the allantoic fluid were examined microscopically, and cultures of the allantoic fluid were made on plates of rabbits' blood agar. When colonies appeared on these plates after an incubation period of 24 or 48 hours at 37°C the individual bacterial species were identified in so far as was possible by appropriate bacteriological and serological procedures. All eggs with living embryos were opened 5 days after inoculation and were examined in a similar manner.

In Table I the data on the incidence of survival and bacterial contamination of the control eggs and those inoculated with the antibiotics are presented. Only 7 (6%) of 120 control eggs survived for 5 days and the great majority died within the first 3 days after inoculation; furthermore all of the eggs that died were found to be heavily contaminated with various bacteria and the deaths can no doubt be largely attributed to bacterial infection. Forty-eight (39%) of

[1] Rose, H. M., Molloy, E., and O'Neill, E., Proc. Soc. Exp. Biol. and Med., 1945, **60**, 23.

[2] Schatz, A., Bugie, E., and Waksman, S. A., Proc. Soc. Exp. Biol. and Med., 1944, **55**, 66.

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TABLE I.

Incidence of Survival and Bacterial Contamination Among Chick Embryos Inoculated with Unfiltered Sputums and Antibiotics.

Day after inoculation	Control				Penicillin				Streptomycin				Penicillin + Streptomycin			
	Survived		Died		Survived		Died		Survived		Died		Survived		Died	
	Contaminated	Sterile	Contaminated	Sterile	Contaminated	Sterile	Contaminated	Sterile	Contaminated	Sterile	Contaminated	Sterile	Contaminated	Sterile	Contaminated	Sterile
1			22				14	2			4	4			2	5
2			58				22	6			13	12			5	8
3			22				10	3			10	2			2	4
4			7				2	5			3	4			4	9
5	4	3	4	0	8	40	4	4	24	36	6	2	19	58	2	2
Total	4	3	113	0	8	40	52	20	24	36	36	24	19	58	15	28

the eggs that received penicillin survived of which 8 were contaminated, mainly with yeasts, and of the 72 that died 52 were contaminated without exception by Gram-negative bacilli. Sixty (50%) of the eggs inoculated with streptomycin survived, but 24 of these proved to be contaminated, chiefly with yeasts, molds, or green-producing streptococci. Seventy-seven (64%) of the eggs inoculated with penicillin together with streptomycin survived of which 19 were contaminated, mainly with yeasts or molds, and it should be noted that among the 43 eggs that died in this group 28 proved to be sterile on culture.

In reference to the results with individual sputum specimens, the frequency with which either one or both chick embryos survived without bacterial contamination, of the individual pairs inoculated with the antibiotics, was as follows: penicillin 26 (43%), streptomycin 28 (47%), penicillin plus streptomycin 43 (70%). With 47 (78%) of the 60 specimens at least one chick embryo survived without bacterial contamination among the 6 inoculated with the antibiotics. Only 3 specimens caused death of all embryos due to bacterial infection and of these 2 were caused by *Ps. aeruginosa* and one by an unidentified coliform organism.

In Table II are presented data indicating the types of bacteria isolated from each of the eggs inoculated with the antibiotics. It will be seen that penicillin is much more effective than streptomycin in preventing bacterial contamination with certain Gram-positive organisms, particularly streptococci. A discrepancy would appear to exist in the case of yeasts and molds, since a larger number of these microorganisms were isolated from the eggs receiving both streptomycin and penicillin than from those inoculated with either agent alone; this may be explained by the fact that the majority of yeasts and molds were isolated from surviving eggs, and the incidence of survival was greatest when both antibiotics were employed. Streptomycin restrained the growth of Gram-negative bacilli in many instances where penicillin was ineffective, particularly with strains of *Pr. vulgaris, Es. coli, Aer. aerogenes,* and unidentified coliforms, but although the incidence

TABLE II.
Strains of Bacteria Isolated from Chick Embyros Inoculated with Unfiltered Sputums and Antibiotics.

	Strepto-cocci	Staphylo-cocci	*Pr. vulgaris*	*Ps. aeruginosa*	*Es. coli*	*Aer. aerogenes*	Coliform	Unidentified gram-negative bacilli	*Sar. lutea*	Chromogenic gram-negative bacilli	Diphtheroids	Yeasts	Molds
Penicillin	1	2	15	15	4	5	13	5	0	0	0	6	0
Streptomycin	14	2	3	10	1	2	7	3	2	0	2	13	1
Penicillin + Streptomycin	1	1	1	9	0	0	2	4	0	2	0	14	3

of infections with *Ps. aeruginosa* was somewhat lower in the eggs that received streptomycin alone or streptomycin plus penicillin, in comparison with those receiving penicillin alone the reduction was not very striking. This frequent failure of streptomycin to inhibit the growth of *Ps. aeruginosa* has been noted by others.[4] It is of considerable interest that Gram-negative bacilli were eradicated by the combined action of streptomycin and penicillin in several instances when either of these compounds alone were ineffective. A possible explanation for this phenomenon resides in the fact that penicillin in large dosage has been shown to exert an antibacterial effect on certain strains of Gram-negative bacilli,[5] and this action may possibly operate synergistically with that of streptomycin when the antibiotics are employed in combination.

Discussion. The results of this study indicate that streptomycin and penicillin together are more effective than either of these agents alone in preventing bacterial contamination and permitting the survival of chick embryos inoculated with unfiltered sputums. The relatively low survival rate of embryos inoculated with penicillin alone, when compared with the results obtained in work previously reported,[1] is probably due to the fact that many sputums were deliberately chosen because they were previously known to contain Gram-negative bacilli. Not only are such organisms ordinarily resistant to the action of penicillin, but they frequently manufacture a substance, penicillinase, which will counteract or nullify the antibacterial effect of this compound.[6] It would appear that, in studies where it is desirable to inoculate unfiltered secretions of the respiratory tract into chick embryos for the intended recovery of viral agents, a helpful procedure would be the use of both penicillin and streptomycin to combat bacterial contamination.

Conclusions. Chick embryos were inoculat-

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ed with unfiltered sputums treated with penicillin and streptomycin singly and in combination. The combination of agents proved to be more effective than either one alone in preventing bacterial contamination and permitting survival of the embryos. Contaminations with Gram-negative bacilli which were resistant to the action of penicillin were controlled in many instances by the addition of streptomycin.

15394

Role of Nitroglycerin in Accelerating Occurrence of the Histamine-Provoked Ulcer.*

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The importance of the vascular factor in the ulcer diathesis has been recently reemphasized. Relative anemia of the mucous membrane of the esophagus, stomach, or duodenum of various experimental animals produced by adrenaline-in-beeswax,[1] fat emboli,[2] and portal hypertension,[3] has been shown to render these structures much more susceptible to histamine-provoked ulcer and/or erosion. In other words, even temporary areas of anemia in the esophageal, gastric or duodenal walls render those areas prone to injury by the gastric juice. The purpose of this presentation is to indicate our observations on the acceleration of the histamine-provoked ulcer in rabbits and dogs, by a drug, nitroglycerin, which dilates the smaller splanchnic vessels.[4]

Methods. These experiments were carried out on rabbits and dogs in 4 series. In the first series, which consisted of rabbits, 0.97 mg nitroglycerin, embedded in beeswax, was implanted daily in the back muscles, in addition to 30 mg of the histamine-in-beeswax mixture prepared after the method of Code and Varco.[5] The animals were sacrificed after varying periods of time. In the second series, dogs were used. Some of these animals received 1.95 mg nitroglycerin embedded in beeswax daily, in addition to 30 mg histamine-in-beeswax intramuscularly each evening. To the remainder of the dogs 30 mg histamine-in-beeswax alone was administered each evening. In each instance the dogs' feed pans were removed and no more food given until the following morning. All the dogs were sacrificed within 2 to 6 days after the beginning of the administration of histamine.

In the third series, the effect of an aqueous solution of 1.3 mg nitroglycerin injected subcutaneously was studied upon dogs with Pavlov and Heidenhain pouches.

In the fourth series recordings of blood pressures were made on anesthetized dogs while the stomach mucosa was exposed and observed for any changes after injection of aqueous nitroglycerin 1.3 mg intramuscularly and intravenously. One series of blood pressure recordings on one animal was recorded on a kymogram by cannulation of a carotid artery. In the other animal used, the blood pressure was measured by femoral artery puncture, by

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† National Cancer Trainee.

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TABLE I.
Production of Ulcer in Rabbits with Both Nitroglycerin and Histamine-in-Beeswax.

Rabbit No.	Daily dose of nitroglycerin in beeswax mg	Daily dose of histamine base mg	Number of injections	Results
1	.97	30	1	Retroperitoneal hematoma probably from rupturing vessel during injection of histamine. Two bleeding points distal to pylorus
2	.97	30	7	Bleeding duodenal ulcers and 1 perforated duodenal ulcer
3	.97	30	9	Bleeding antral ulcer
4	.97	30	4	Perforating and bleeding duodenal ulcer
5	.97	30	4	Bleeding duodenal ulcer
6	.97	30	11	No ulcer
7	.97	30	2	"
8	.97	30	2	Perforating antral ulcer
9	.97	30	2	"
10	.97	30	25	Ulcer at junction of esophagus and stomach
11	.97	30	26	No ulcer
12	.97	30	26	Antral ulcer
Summary: Nine out of 12 animals developed ulcers				
Controls				
A	.97	0	25	No ulcers
B	.97	0	25	"
C	.97	0	25	"
1 H	0	20	31	"
2 H	0	20	31	"
3 H	0	20	31	"

TABLE II.
Production of Ulcer in Dogs with Both Nitroglycerin and Histamine-in-Beeswax.

Dog No.	wt lb	Daily dose of nitroglycerin in beeswax mg	Daily dose of histamine base mg	No. of injections	Results	No. of days before sacrifice
1	17	1.95	30	2	Large perforating duodenal ulcer; multiple bleeding erosions in antrum; antral ulcers	3
2	12	1.95	30	3	Multiple bleeding antral ulcers	4
3	23	1.95	30	6	Duodenal ulcer	7
4	15	1.95	30	4	Perforating duodenal ulcer; cardio-esophageal ulcer	5
Controls						
5	47	0	30	3	No ulcer	4
6	24	0	30	6	"	7
7	52	0	30	6	"	7

Summary:
Dogs receiving both nitroglycerin and histamine—% with ulcers—100%
Dogs receiving no nitroglycerin but histamine—% with ulcers—0%

use of a mercury manometer.

Experiments. Series I. Twelve rabbits were used in this series. Both the nitroglycerin and histamine were administered for periods varying from one to 26 days.

Series II. Seven dogs were used in this series. Four dogs received both nitroglycerin and histamine for periods varying from 2 to 6 days. The 3 remaining dogs were given histamine alone for periods of 3 to 6 days.

Series III. Aqueous nitroglycerin was injected subcutaneously into the Pavlov pouch and Heidenhain pouch dog. Collections were then taken and gastric analyses for volume

and free and total HCl were made.

Series IV. Two dogs were used in this series and aqueous nitroglycerin was injected intramuscularly and in one instance intravenously. If the blood pressure showed no change within 15 minutes, another dose was given. This was repeated for a total of 3 doses intramuscularly and one intravenous dose in one dog and 3 doses intramuscularly in the other.

Results. The results of Series I are tabulated in Table I. Nine out of 12 rabbits receiving both nitroglycerin and histamine had ulcers and/or erosions. There were no ulcers in the control animals.

Table II represents the results in Series II. In the dogs receiving both nitroglycerin and histamine-in-beeswax, ulcers were observed in an equal period of time as in animals receiving histamine-in-beeswax alone.

In the gastric secretion studies (Series III) no stimulation of either free HCL or volume by aqueous nitroglycerin was observed. Three readings on the Pavlov pouch dog and 3 on the Heidenhain pouch dog were made.

The studies on blood pressure revealed no significant findings. However, the changes in the exposed gastric mucosa were certainly significant. After the initial dose of nitroglycerin a definite blanching of circular areas of mucosa took place. This occurred within 2 minutes after the drug had been given. As time went on, this blanching became even more marked. This marked pallor lasted during the entire experiment.

Comment. It is apparent from these experiments that nitroglycerin-in-beeswax aids and abets the ulcer diathesis. It has been shown that the primary action of the nitrites is on the venous side of the circulation. There is a decreased venous tone in both the splanchnic and peripheral areas. The vasomotor responses of the arterioles to certain local and reflex stimuli are maintained in the presence of decreased venous tone.[6] It has also been shown that vascular changes observed as variations in color of the exposed gastric mucous membrane actually reflect changes in blood flow.[7] The blanching of the gastric mucosa is thus an indication of decreased blood flow. This occurrence, coupled with venous pooling, may result in such a low blood flow to the mucosa that a dangerous degree of anoxemia is produced, which condition, if maintained over a long period of time, increases the ulcer diathesis. We have been unable to produce ulcer in rabbits, with histamine alone.[8] However, as is indicated herein, bleeding ulcers and erosions follow regularly when histamine administration is accompanied by implantation of nitroglycerin-in-beeswax.

The uniform production of ulcer in dogs within 2 to 6 days following a daily injection of both nitroglycerin and histamine emphasizes the importance of the vascular factor in the ulcer diathesis.

Summary. Nitroglycerin - in - beeswax is shown to aid and abet the histamine-provoked ulcer in dogs and rabbits. The effect of nitroglycerin is believed to be due to its venous pooling property; and this action, coupled with normal arteriolar reflex contractile responses, leads, after prolonged administration, to resultant areas of impaired circulation in the mucous membrane of the esophagus, stomach and duodenum. These areas of lowered resistance are then subjected to acid-peptic digestion with resultant ulcer and/or erosion formation.

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15395 P

Use of Bacteriostatic Agents in Preparation of Seed Cultures of Psittacosis Virus.*

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From Camp Detrick, Maryland.

Previous experiments[1] have indicated that psittacosis virus (strain 6 BC) is resistant to the action of streptomycin when grown in roller tube tissue cultures of minced chick embryo tissue. Experiments[1] also indicated that this virus would survive in as much as 50 mg % sodium sulfadiazine in the buffered salt solution-serum ultrafiltrate fluid[2] containing a small amount of dead chick embryo tissue when kept at room temperature for 5 days although it was inhibited by sulfadiazine when actively growing in tissue cultures and eggs.[1] The resistance of the virus to these 2 agents, suggested their use in the control of contamination in seed cultures where the virus is not actively multiplying.

Preparation of Seed Cultures. Eight-day-old embryonated eggs were injected by the yolk sac route with 0.25 ml of a 10^{-6} dilution in nutrient broth of yolk sacs infected with psittacosis virus (strain 6 BC). These embryos died on the fourth day, at which time yolk sacs were harvested and made into a 20% suspension in broth by shaking with glass beads for ½ hour at 0°C. This suspension was stored overnight at 0°C.

The 20% suspension of infected yolk sacs was divided into 2 parts. Lot I was diluted to 10% by the addition of an equal volume of nutrient broth. An equal amount of nutrient broth containing 50 mg % sodium sulfadiazine and 250 u/ml of streptomycin was added to Lot II to give a final concentration of 25 mg % sodium sulfadiazine and 125 u/ml streptomycin. These seed cultures were sealed in glass vials, shell-frozen, and stored in the CO_2 ice box.

Repeated simultaneous comparative titrations were made on Lots I and II by injecting serial dilutions of the virus suspensions in nutrient broth as follows: 0.03 ml intracerebrally and 0.25 ml intravenously in 18-20 g mice and 0.25 ml into the yolk sacs of 6-day-old embryonated eggs. There were no significant differences in the titers of the virus obtained on Lots I and II by any of the 3 methods used. Titers were calculated as LD_{50} by the method of Reed and Muench.[3]

These results indicated that 25 mg % sodium sulfadiazine and 125 u/ml of streptomycin can be added to 10% yolk sac seed cultures of psittacosis virus (strain 6 BC) as a safeguard against possible contamination without any deleterious effect on the titer of the virus when sealed in glass vials and stored in a CO_2 ice box.

Preliminary experiments also indicated that the use of these bacteriostatic agents in diluting fluids (50 mg % sodium sulfadiazine and 250 u/ml of streptomycin) is of considerable value in the isolation of psittacosis virus by intracerebral injection in mice from such contaminated sources as urine, feces and soil.

Summary. The addition of 25 mg % sodium sulfadiazine and 125 u/ml of streptomycin to yolk sac seed cultures of psittacosis virus stored with carbon dioxide ice had no effect on the titer of the virus.

* Studies conducted at Camp Detrick, Md., from March to May, 1945.

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15396 P

An Organic Antimony Compound with Curative and Prophylactic Activity in Experimental Trypanosomiasis.

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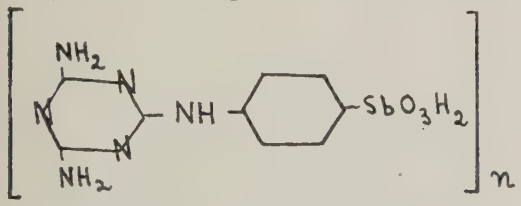
Two types of compounds, both metal free, are known to have a prophylactic effect in trypanosomiasis: the polysulfonated ureide "naphuride" (Synonyms: Bayer 205, Germanine, Moranyl, etc.) and amidines such as pentamidine and stilbamidine.

A third type of prophylactic compound, of the organometallic type, has now been obtained: a sodium salt derived from *p*-(2,4-diamino-1,3,5-triazinyl-6) aminophenylstibonic acid. (Synonym: melaminylphenylstibonic acid) where n is larger than 3 and connotes a



degree of polymerization, which will be discussed elsewhere.

This water soluble compound, containing quinquevalent antimony, was tolerated by 56 out of 56 mice in a single intraperitoneal dose of 2.5 g/kg. A single intraperitoneal dose of 0.0125 g/kg cured definitely without relapse (blood smears examined every second day for 40 days), all of 34 mice from a *T. equiperdum* infection which killed all of 10 untreated control mice within 3 to 5 days. The treatment was performed 24 hours after an intraperitoneal infection, at a time when the experimental animals showed at least one trypanosome per hundred red blood cells. The therapeutic index amounts thus to 200.

The prophylactic effect against a single infection is brought out by the following screening experiment: 5 mice are treated with a single intraperitoneal dose of 0.050 g/kg, polymerized sodium *p*-melaminylphenylstibonate (corresponding to 1/50th of the 100% tolerated dose), and infected 69 days later, together with 5 control mice with *T. equiperdum*. All controls die, within 5 days, of acute trypanosomiasis, whereas all pretreated animals survive and do not take the infection, as proved by daily negative blood examinations continued over 40 days. In this case the mechanism of the prophylactic activity seems to be based on the chemical effect of a drug which is resorbed and eliminated so slowly that a sufficient trypanocidal concentration is maintained in the organism over a considerable length of time. This theory is substantiated by the following observation: The pk of the compound is such that the free stibonic acid is precipitated by CO_2 from solutions of its sodium salt. After intraperitoneal and intramuscular injections of the sodium salt a white deposit of the free acid can be readily seen for many weeks on the surface of the peritoneum and between the muscle fibers.

The prophylactic effect against repeated infection is demonstrated in the next experiment:

105 mice are treated with one single in-

TABLE I.

% animals protected	Days after single treatment	No. of test infections
100	29	1
100	36	2
100	43	3
100	50	4
98	57	5
98	64	6
97	71	7
95	78	8
95	85	9
69	92	10
57	99	11
39	106	12
28	114	13
22	121	14
15	128	15
15	135	16
9	146	17
6	155	18
0	164	19

traperitoneal injection of 0.3 g/kg of polymerized sodium *p*-melaminylphenylstibonate. One month later the animals are given a *T. equiperdum* infection which kills the untreated controls without exception in 3 to 5 days. The test infection is repeated at weekly intervals on the surviving mice found to be free of trypanosomes. Untreated controls are set up at each infection and die without exception within 3 to 5 days. Blood examinations are performed daily on all animals. The results are summarized in Table I.

It is noteworthy that the mice finally breaking down under multiple infections show an atypical form of trypanosomiasis, differing basically from the classical acute blood infection characterized by the number of trypanosomes increasing continuously and progressively until death occurs. The pretreated animals present a chronic infection, of the human and rabbit type, with parasites appearing in and disappearing from the blood at irregular intervals. The post mortem of these chronic cases shows, invariably, an enormously enlarged spleen up to 10 times the weight and size of the normal spleen.

In this last experiment with repeated infections the mechanism of the prophylactic effect may possibly include an immunological factor.

Summary. A new heterocyclic stibonic acid is described, having a pronounced prophylactic effect in the *T. equiperdum* infection of the mouse and which cures this infection with a single intraperitoneal dose representing 1/200th of the maximum (100%) tolerated dose.

15397

Concentration of Chloride, Sodium and Potassium in Blood of Guinea Pigs Treated with Desoxycorticosterone Acetate.*

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The fibromatogenic effect of estrogens is known from the work of Nelson,[1] Lipschutz and Iglesias.[2,3] Subsequently the antifibromatogenic effect of steroids such as progesterone, testosterone, dihydrotestosterone and desoxycorticosterone has been demonstrated by Lipschutz and others.[4]

Desoxycorticosterone offers special interest clinically because the sex organs were not affected by antifibromatogenic quantities of this steroid, as was the case with the androgens. However, several clinicians[5,6] have described toxic actions in patients treated with therapeutic doses of desoxycorticosterone acetate. Changes in the concentration of the ions of the blood have been found in these patients. Selye and his collaborators[7,8] have induced important modifications of the ionemia with

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TABLE I.

Forty-eight Castrated Female Guinea Pigs with Subcutaneously Implanted Small Tablets of Diethylstilbestrol and Desoxycorticosterone Acetate. Necropsy at 113 to 115 Days after Castration and Implantation.

Groups	Estrogen per day avg µg	Desoxycorticosterone per day avg µg	Wt of uterus g	Fibrous tumoral effect F.T.E. avg units§	No. of animals total	No. of animals reaching avg F.T.E. of stilb. group	No. of tumoral marks of classes 2 and 3 per animal§
I	(a)	(b)	(c)	(d)	(e)	(f)	(g)
No treatment	0	0	—	0	16	0	0
II							
Estrogen only	13.4 (9-18)*	0	7.0 (5.0-9.0)†	5.5	10	5	1.6
III							
Estrogen and desoxycorticosterone	15.0 (9.5-29)	222 (159-275)	3.1 (1.7-4.0)‡	1.7	11	0	0.1
IV							
Only desoxycorticosterone acetate	0	217 (131-265)	0.7 (0.3-1.2)	0	11	0	0

* ( ) Range.
† One 13.0 g. ‡ One 7.7 g.
§ For the system of classification and units see Lipschutz *et al.*[12,13]

large quantities of desoxycorticosterone acetate. But Lipschutz and Vargas[9] were unable to obtain any toxic effect with quantities of desoxycorticosterone acetate by which estrogen-induced abdominal fibroids were prevented in the guinea pig.

In the present paper we refer to the determination of chloride, sodium and potassium in the blood of guinea pigs treated during 113 days simultaneously with fibromatogenic quantities of diethylstilbestrol and quantities of desoxycorticosterone acetate sufficient to prevent estrogen-induced fibroids. The determinations of chloride, sodium and potassium were made in the plasma. Blood was obtained by puncture of the heart. Lithium oxalate was used as an anticoagulant. Determinations were made in duplicated samples. Volhard's classical procedure with the addition of nitrobenzene was used for the determination of chloride. Microdetermination of sodium was made by the method of Marenzi and Gerschman,[10] and that of potassium was made according to the method of Kramer (quoted from Corona.[11])

Blood samples were taken 45, 73 and 101 days after the subcutaneous implantation of tablets. The tablets of diethylstilbestrol contained 40% estrogen and 60% cholesterol. The tablets of desoxycorticosterone acetate were prepared without any admixture.

Table I summarizes our results with necropsies of treated animals and controls. As seen from the table the quantities of desoxycorticosterone were sufficient to inhibit to a considerable degree both the estrogen-induced growth of the uterus and the fibromatogenic effect.

Results of the determination of chlorides, sodium and potassium are given in Table II.

There were no significant differences between the values obtained under the influence of estrogens or desoxycorticosterone. Sodium

9 Lipschutz, A., and Vargas, L., *Lancet*, 1941, **1**, 568.

10 Marenzi, A., and Gerschman, R., *Rev. de la Soc. Arg. Biol.*, 1943, **7**, 381.

11 Corona, L., *Química normal y patológica de la sangre*, 3a. ed., Ercilla, Santiago (Chile), 1942.

12 Lipschutz, A., Bellolio, P., Chaume, J., and Vargas, L., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 164.

13 Lipschutz, A., and Maass, M., *Cancer Research*, 1944, **4**, 18.

TABLE II.
mg %.

Days after implantation of tablets	Chloride				Sodium			
	I Contr.	II Estrog.	III Estr.-Deso.	IV Deso.	I Contr.	II Estrog.	III Estr.-Deso.	IV Deso.
45	360	329	346	318	277	278	263	274
73	352	351	376	380	277	279	287	290
101	381	374	373	368	278	278	314	295

Days after implantation of tablets	Potassium			
	I Contr.	II Estrog.	III Estr.-Deso.	IV Deso.
45	31.4	25.5	22.7	31.7
73	34.0	27.3	28.0	29.9
101	19.7	21.8	19.8	26.9

was rather high in Groups III and IV, but the average was not higher than the maximal values in Groups I and II without desoxycorticosterone. There were important variations of potassium, but they were not related to the administration of desoxycorticosterone.

Summary. A group of castrated female guinea pigs was treated with fibromatogenic quantities of diethylstilbestrol; another group was treated simultaneously with diethylstilbestrol and quantities of desoxycorticosterone acetate sufficient to prevent estrogen-induced abdominal fibroids. The average fibrous tumoral effect diminished by the action of desoxycorticosterone from 5.5 to 1.7 units. Concentration of chloride, sodium and potassium in the blood of the groups treated with diethylstilbestrol, desoxycorticosterone or both was within the same range as in animals not treated. These results show that the antifibromatogenic quantity of desoxycorticosterone acetate is smaller than those doses which might interfere with the concentration of chloride, sodium or potassium in the blood.

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Antigenic Differences Between Strains of Scrub Typhus as Demonstrated by Cross-Neutralization Tests.

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The question of whether antigenic differences occur among strains of scrub typhus is important not only from an epidemiological point of view but also with regard to the development of a vaccine for its control. Epidemiologic studies have indicated a marked variation in the severity of outbreaks of this disease in different geographical areas.[1,2] Furthermore, there have been undocumented rumors from medical officers returning from the southwest Pacific area that individuals recently recovered from the disease in one locality have been reinfected in another. This

* Member, United States of America Typhus Commission.

[1] Blake, F. G., Maxcy, K. F., Sadusk, J. F., Jr., Kohls, G. M., and Bell, E. J., *Am. J. Hyg.*, 1945, **41**, 243.

[2] Kohls, G. M., Armbrust, C. A., Irons, E. N., and Philip, C. B., *Am. J. Hyg.*, 1945, **41**, 374.

would imply that the immunity conferred by the first infection was inadequate to prevent the second. However, such an inadequacy might be attributed as easily to relative pathogenicity of strains as to antigenic differences.

Although experimental evidence indicates that laboratory animals which survive infection with one strain are completely immune to infection with other strains,[1,3,4] this by itself is not conclusive proof of the complete antigenic identity of such strains. For example, guinea pigs recovered from an infection with either epidemic or murine typhus are resistant to infection not only with the homologous agent but also with the heterologous agent. Nevertheless, antigenic differences between the agents are readily demonstrable by serological means[5] and the vaccination of animals with one of them gives little protection against infection with the other.[6] A similar relationship probably exists between strains of scrub typhus. Thus, Topping[3] showed that infection with the Karp or Gilliam strain conferred immunity to the heterologous as well as homologous strains while Bengtson[7] presented evidence of their antigenic differentiation. She found[7] that sera from patients and animals infected with the Karp or Gilliam strain fixed complement strongly with the homologous antigen but only weakly with the heterologous. Not all strains are thus separable, for Smadel, Rights and Jackson[4] found the Imphal and Calcutta strains to be indistinguishable in cross-immunity and complement fixation tests.

The data to be presented indicate that differentiation between certain strains of *R. orientalis* can be demonstrated by means of cross-neutralization tests.

Strains of Rickettsia orientalis. Three strains of *R. orientalis* were used in the present experiments. The Kostival strain[1] was isolated in the Dobadura area, New Guinea, from a case of naturally acquired infection. The Host 21 strain,[1] recovered from mites *(T. fletcheri)* taken from a wild caught bandicoot *(Echymipera cockerelli)*, was also isolated in the Dobadura area, New Guinea. Both strains have been maintained by passage in mice and in yolk sacs of embryonated eggs. The Seerangayee strain[8] was isolated by Lewthwaite and Savoor from a case occurring in southern Malaya and has since been maintained in mice and guinea pigs.

Preparation of Sera. Kostival and Seerangayee antisera were prepared by inoculating rabbits with a 10 or 20% suspension of liver and spleen from infected hamsters. 5.0 cc were injected intraperitoneally and 2.0 cc subcutaneously on 2 occasions a week apart. Two weeks after the second injection the rabbits were bled and their sera pooled. The Host 21 antiserum consisted of pooled sera from guinea pigs which had received a single intraperitoneal injection of 2.0 cc of a 10% suspension of infected hamster liver and spleen. The guinea pigs were exsanguinated one month after the inoculation.

Neutralization Tests. The technic used for the performance of neutralization tests consisted of adding undiluted experimental and control sera to equal volumes of 10-fold dilutions of previously standardized stock rickettsial suspensions. These suspensions were prepared from infected yolk sacs or mouse tissues and stored at —70°C. The dilutions of infectious material were $10^{-1.7}$, $10^{-2.7}$, etc., so that after the addition of the serum the final dilutions would be 10^{-2} (1:100), 10^{-3} (1:1000), etc. After mixing, the tubes were placed in ice water and 0.2 cc amounts of each of the serum-virus mixtures were injected intraperitoneally into 5 to 10 mice. Mice were observed daily for 21 days at which time the survivors were discarded. The LD_{50} titers of the virus in the experimental and control sera were determined by the method of Reed and Muench, the values expressed as the logarithm of the reciprocal

3 Topping, N. H., *Pub. Health Rep.*, 1945, **60**, 945.

4 Smadel, J. E., Rights, F. L., and Jackson, E. B., *J. Exp. Med.*, 1946, **83**, 133.

5 Plotz, H., Wertman, K., and Bennett, B. L., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 76.

6 (a) Ventemillas, F., *J. Immunol.*, 1939, **36**, 339; (b) Wertman, K., unpublished data.

7 Bengtson, I. A., *Pub. Health Rep.*, 1945, **60**, 1483.

8 Lewthwaite, R., and Savoor, S. R., *Brit. J. Exp. Path.*, 1936, **17**, 1.

TABLE I.
Titration of Kostival Strain in the Presence of Homologous and Heterologous Rabbit Immune Serum.

Immune serum	Dilution of rickettsiae 10-2	10-3	10-4	10-5	10-6	10-7	10-8	Infectious titer† (log)	Neutralizing index† (log)
Kostival rabbit No. 2	0/6*	0/6	2/6	1/6	0/6	—	—	2.0	4.2
Seerangayee rabbit No. 1	6/6	6/6	3/6	4/6	2/6	—	—	5.1	1.1
Normal rabbit	6/6	5/6	6/6	5/6	3/6	3/6	0/6	6.2	—

* Numerator = number of mice dying.
Denominator = number of mice inoculated.
† Titers are recorded as the logarithm of the reciprocal of the dilution of virus producing 50% mortality. The index is recorded as the difference between the logarithmic exponents of the titers of the virus in the experimental and control sera.

TABLE II.
Preliminary Cross-Neutralization Tests Between Kostival, Seerangayee and Host 21 Antisera and Their Respective Agents.

Exp. No.	Infectious agent	Immune serum	Infectious titer in Test group (log)	Infectious titer in Control group (log)	Neutralizing index (log)
1	Kostival	Kostival rabbit No. 1	2.0	5.8	3.8
2	Host 21	" "	4.0	3.4	—0.6
3	Kostival	" "	2.0	6.5	4.5
	Host 21	" "	5.4	6.2	0.8
4	Kostival	Kostival rabbit No. 2	2.0	6.2	4.2
	"	Seerangayee rabbit No. 1	5.1	6.2	1.1
5	Seerangayee	" "	2.0	6.3	4.3
6	Host 21	Host 21 GP No. 1	2.2	6.5	4.3
	Kostival	" "	3.3	6.5	3.2

See explanatory notes of Table I.

of the calculated dilution producing mortality in 50% of the animals inoculated. The neutralizing index was taken as the difference between the logarithmic exponents of the experimental and control titers. An example of such a titration is given in Table I. The detailed data are from Experiment 4, which is summarized in Table II.

Results. The results of a series of exploratory experiments are summarized in Table II. In Experiment 1 the Kostival antiserum was shown to neutralize the homologous agent. In Experiment 2, however, this serum failed to neutralize the heterologous strain (Host 21). A fresh Host 21 suspension was prepared and used in Experiment 3. Here the Kostival antiserum again neutralized the homologous strain but failed to neutralize the heterologous strain. In Experiment 4 a new Kostival antiserum was utilized and compared with a Seerangayee antiserum with results similar to those obtained above, namely, the homologous serum neutralized the agent while the heterologous serum was relatively inert. That this was not due to lack of antibodies in the Seerangayee antiserum is indicated by Experiment 5 where excellent homologous neutralization was demonstrated. In Experiment 6, however, a somewhat different situation was encountered in that the Host 21 guinea pig antiserum, which neutralized its own virus to high titer, also neutralized the heterologous Kostival strain.

Subsequently, a series of experiments were performed in which 3 of the antisera used in the earlier studies were tested against the 3 agents. The results summarized in Table III indicate that, as before, the Kostival antiserum was effective against the Kostival strain only. Similarly, the Seerangayee antiserum was relatively ineffective against both the heterologous strains. In contrast, the Host 21 antiserum was capable of neutralizing all 3 strains, in fact, giving a higher index against

TABLE III.
Cross-Neutralization Tests Between Kostival, Seerangayee and Host 21 Antisera and Their Respective Agents.

Exp. No.	Infectious agent	Immune serum	Infectious titer in Test group (log)	Infectious titer in Control group (log)	Neutralizing index (log)
7	Kostival	Kostival rabbit No. 2	3.3	6.0	2.7
	"	Seerangayee rabbit No. 1	5.1	6.0	0.9
	"	Host 21 GP No. 1	3.7	6.0	2.3
8	Seerangayee	Kostival rabbit No. 2	6.2	7.2	1.0
	"	Seerangayee rabbit No. 1	3.6	7.2	3.6
	"	Host 21 GP No. 1	3.5	7.2	3.7
9	Host 21	Kostival rabbit No. 2	5.4	5.7	0.3
	"	Seerangayee rabbit No. 1	4.2	5.7	1.5
	"	Host 21 GP No. 1	3.4	5.7	2.3

See explanatory notes of Table I.

the Seerangayee virus than against its own Host 21 strain. The significance of this fact remains to be determined. It is to be noted that all homologous serum titers had dropped considerably since the experiments recorded in Table II. It is possible that this was due to technical details in performing the test which have escaped our notice, or that it was related to the fact that the sera were stored at 4°C. Workers in the virus field recognize that the antibody titers of certain sera diminish when stored at 4°C. Attempts to restore the activity of scrub typhus sera by the addition of freshly drawn rabbit or guinea pig serum were unsuccessful; this was in contrast to the results of Morgan[9] and Whitman[10] in reactivation experiments with Western equine encephalitis antisera.

Discussion. The experiments reported above indicate that distinct differences can be demonstrated between certain strains of *R. orientalis* by means of cross-neutralization tests. It is of interest that while the Kostival strain from the Dobadura area of New Guinea produced a serum which had little capacity to neutralize the Host 21 strain obtained from the same area, yet the serum from guinea pigs inoculated with Host 21 had considerable capacity to neutralize the Kostival agent. A similar situation existed between the Seerangayee strain from Malaya and the Host 21 strain. Several possibilities arise to explain such a phenomenon. In the first place, the origin of the strains may be responsible for these differences. Host 21 was recovered from mites taken from a wild caught bandicoot *(Echymipera cockerelli)* whereas Kostival and Seerangayee were isolated from human cases. Perhaps the parent mite strains are antigenically more complete than those isolated from humans. Secondly, both the Kostival strain and the Seerangayee strain had been passaged either through chick embryo (Kostival) or guinea pigs (Seerangayee) with much greater frequency than the Host 21 strain. This may have resulted in a partial loss of antigenic structure. Thirdly, the Host 21 antiserum was prepared in guinea pigs, whereas the other 2 antisera were obtained from rabbits. Although experiments have indicated no difference between normal guinea pig and normal rabbit serum in virucidal properties, it is possible that this difference in animal species may play an important part in the specificity of the sera produced.

Summary and Conclusions. The antigenic relationship of 3 strains of *R. orientalis* was studied by means of cross-neutralization tests. Antiserum produced in rabbits to the Kostival or Seerangayee strains neutralized the homologous strain only. However, antiserum produced in guinea pigs to the Host 21 strain showed a broader coverage.

[9] Morgan, I. M., *J. Immunol.*, 1945, **50**, 359.

[10] Whitman, L., to be published.

15399 P

A Toxic Substance Associated with the Gilliam Strain of *R. orientalis.*

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A substance toxic for mice occurs in yolk sac tissues infected with *R. mooseri*[1] and *R. prowazeki.*[2] These related toxins are neutralized by typhus antisera,[1,2] but are not immunologically identical.[3,4] The present report describes the occurrence of a specific toxic material in yolk sacs infected with the Gilliam strain of *R. orientalis.*

Materials and Methods. The Gilliam strain,[5] received from Dr. N. H. Topping, was maintained by serial passage in yolk sacs of 7-day-embryonated eggs which were incubated at 35°C after inoculation. Yolk sacs infected with the 25th to 36th egg passage yielded toxic material. The methods of preparation and testing were as follows: Sacs of embryos moribund on the sixth to ninth day after inoculation were harvested, and smears, stained by a modified Macchiavello's technic, were examined microscopically. Those containing 25-100 recognizable rickettsiae per field were pooled and a 20% suspension prepared in sterile skimmed milk. Following low speed centrifugation, ½, ¼ and ⅛ dilutions of the supernatant fluid were prepared in 0.9% NaCl solution containing 10% normal rabbit serum. 0.5 cc of each dilution was injected intravenously into each of 5 mice. Deaths were recorded up to 18 hours when survivors were discarded. Toxin-neutralization tests were done as follows: A ½ dilution of a 20% suspension of freshly harvested infectious sacs was tested for toxin in 5 mice; if 4 died within 2 hours, the material was considered suitable. Serial 2-fold dilutions of sera to be tested were then prepared in 10% normal serum-NaCl solution. These were mixed with equal volumes of the stock 20% suspension of sacs which had been stored meanwhile at 0°C. Intravenous injection of mice with 0.5 cc amounts of the mixtures was begun immediately and usually completed within an hour. A control titration of the toxic suspension was performed at the end of the test. Toxin-neutralization tests were done with pooled sera of guinea pigs and serum of one patient recovered from infection with the Gilliam strain, and with pooled sera of rabbits hyperimmunized with splenic tissue of mice dead from infection with this strain. Antisera against the Karp,[5] Kostival,[6] Host 21,[6] Seerangayee,[7] Volner,[8] Imphal,[9] Wild Rat No. 2,† and CBI Rat† strains of scrub typhus were also tested for antitoxin. These had been shown in other experiments[10] to be capable of protecting mice from death caused by infection[11] with the homologous strain.

Results. Mice injected intravenously with toxic preparations appeared normal for 1 to 1½ hours, then developed dyspnea and weak-

* Member of United States of America Typhus Commission.

1 Gildemeister, E., and Haagen, E., *Deut. Med. Wchnschr.*, 1940, **66**, 878.

2 (a) Bengtson, I. A., Topping, N. H., and Henderson, R. G., *National Institute of Health Bull.* No. 183, U. S. Government Printing Office, Washington, 1945, pp. 25-29; (b) Henderson, R. G., and Topping, N. H., *ibid.*, pp. 41-56.

3 Hamilton, H. L., *Am. J. Trop. Med.*, 1945, **25**, 391.

4 Groupe, V., and Donovick, R., *Science*, 1946, **103**, 330.

5 Bengtson, I. A., *Pub. Health Rep.*, 1945, **60**, 1483.

6 Blake, F. G., Maxcy, K. F., Sadusk, J. F., Jr., Kohls, G. M., and Bell, E. J., *Am. J. Hyg.*, 1945, **41**, 243.

7 Lewthwaite, R., and Savoor, S. R., *Brit. J. Exp. Path.*, 1936, **17**, 1.

8 Philip, C. B., Woodward, T. E., and Sullivan, R. R., *Am. J. Trop. Med.*, 1946, **26**, 229.

9 Smadel, J. E., Rights, F. L., and Jackson, E. B., *J. Exp. Med.*, 1946, **83**, 133.

† Isolated in New Guinea and Burma by members of the U. S. A. Typhus Commission.

10 Smadel, J. E., and Bennett, B. L., to be published.

11 Bell, E. J., Bennett, B. L., and Whitman, L., Proc. Soc. Exp. Biol. and Med., 1946, **62**, 134.

TABLE I.
Neutralization of Toxin of *R. orientalis* (Gilliam Strain) by Hyperimmune and Convalescent Scrub Typhus Sera.

Toxin		Antisera			Results	
Material	Dilution	Strain	Source	Dilution	Death of mice*	Antitoxin titer (dil. of serum)
	1/10	None			17/20	
	1/20	,,			1/10	
	1/40	,,			0/5	
	1/10	Gilliam	rabbit	1/10	0/5	
	,,	,,	,,	1/40	0/5	
	,,	,,	,,	1/160	0/5	1/640
	,,	,,	,,	1/640	0/5	
	,,	,,	guinea pig	1/10	0/5	
	,,	,,	,,	1/40	1/5	
	,,	,,	,,	1/160	0/5	1/640
	,,	,,	,,	1/640	0/5	
	,,	,,	human	1/5	1/5	
	,,	,,	,,	1/10	0/5	1/20
	,,	,,	,,	1/20	0/5	
Gilliam yolk sac 31st passage	,,	Karp	guinea pig	1/10	1/5	
	,,	,,	,,	1/20	0/5	1/40
	,,	,,	,,	1/40	0/5	
	,,	Wild rat No. 2	rabbit	1/10	1/4	
	,,	,,	,,	1/20	2/5	1/10
	,,	,,	,,	1/40	5/5	(partial 1/20)
	,,	Imphal	,,	1/10	1/5	
	,,	,,	,,	1/20	3/5	1/10
	,,	,,	,,	1/40	4/5	(partial 1/20)
	,,	Volner	,,	1/10	5/5	
	,,	,,	,,	1/20	4/5	Negative
	,,	,,	,,	1/40	5/5	
	,,	CBI rat	,,	1/10	5/5	
	,,	,,	,,	1/20	5/5	,,
	,,	,,	,,	1/40	3/5	
	,,	None			11/15	
	1/20	,,			9/15	
	1/40	,,			0/15	
	1/10	Gilliam	rabbit	1/320	0/5	
	,,	,,	,,	1/640	0/5	1/640
	,,	,,	,,	1/1280	2/5	(partial 1/1280)
Gilliam yolk sac 36th passage	,,	Seerangayee	,,	1/10	0/5	
	,,	,,	,,	1/20	4/5	1/10
	,,	,,	,,	1/40	4/5	
	,,	Host 21	guinea pig	1/10	5/5	
	,,	,,	,,	1/20	4/5	Negative
	,,	,,	,,	1/40	4/5	
	,,	Kostival	rabbit	1/10	4/5	
	,,	,,	,,	1/20	5/5	,,
	,,	,,	,,	1/40	5/5	

* Numerator indicates number of mice which died and denominator indicates the number of mice in each group.

ness followed rapidly by prostration, cyanosis, convulsions and death. Practically all deaths occurred within 3 hours. Certain mice affected by the toxin recovered and looked healthy for several days; all subsequently died in 4-6 days with the usual signs of infection[9] and with numerous rickettsiae in their spleens and lungs.

The toxic factor occurred with some regularity in our preparations although the titers were never high. In 21 experiments 5 suspensions killed the majority of mice receiving a 1/20 dilution and 8 killed at 1/10, while 8 were non-lethal at the latter dilution. The toxicity of a suspension generally diminished after freezing and storage at —70°C or —20°C for a few hours but was consistently unaffected by storage at 0°C for 3-4 hours. These same general methods have failed in our hands to demonstrate toxicity in yolk sacs heavily infected with the Imphal, Calcutta,[9] Karp, Kostival and Host 21 strains of *R. orientalis*. Whether this indicates inherent differences in these strains or that growth is not profuse enough in eggs to produce detectable toxin remains to be determined.

Sera of normal men, rabbits and guinea pigs did not neutralize the toxin. In contrast, a 1/640 dilution of anti-Gilliam rabbit or guinea pig serum neutralized a lethal dose of toxin, and the patient's serum had a titer of 1/20, Table I. Antisera against only 4 of the 8 heterologous strains of *R. orientalis* contained even small amounts of antitoxin, yet each serum was known to protect mice against death by infection with at least 1,000 lethal doses of its homologous strain. Thus, results of toxin-antitoxin tests contribute to the other types of evidence[5,11] regarding immunological differences among strains of *R. orientalis*. Sera of guinea pigs convalescent from epidemic typhus, murine typhus, or spotted fever failed to neutralize the Gilliam toxin; their specific complement fixing titers[12] were 1/240, 1/320, and 1/320, respectively.

Conclusion. Yolk sacs of embryonated eggs infected with the Gilliam strain of *R. orientalis* contain a specific toxin lethal for mice. The toxic substance is readily neutralized by Gilliam antiserum, but antisera against 8 other strains of *R. orientalis* contain only small amounts of antitoxin or none.

[12] Plotz, H., Wertman, K., and Bennett, B. L., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 76.

15400

Effect of Distention of Jejunum upon Tonicity of the Cardia of the Dog.

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This study was undertaken to determine if there are any extrinsic reflex effects upon the tonus and motility of the cardia during distention of the jejunum.

Methods. Records were obtained from 4 dogs over a period of 3 months. A modification and extension of the method outlined by Zeller and Burget[1] for recording motility of the cardia in the unanesthetized dog was used. A jejunal Thiry fistula was prepared in each dog, then an esophagostomy was made following recovery from the first operation.

About a week after the esophagostomy was performed, the training of the dogs was begun. They were trained to lie quietly on a table with the balloons in position while records of motility were obtained. Three balloons were in place and simultaneous tracings made on a smoked drum. The upper balloon was a 2 cm segment of condom tied over a rather rigid 14 Fr. catheter and inserted about 4 or 5 cm into the esophageal fistula and tied in place. It was attached to a

[1] Zeller, W., and Burget, G. E., *Am. J. Dig. Dis.*, 1937, **4**, 113.

mercury manometer. The balloon for recording cardial activity consisted of a 3 cm segment of condom enclosing soft sponge rubber on the end of a 16 Fr. catheter. The soft sponge rubber served the purpose of keeping the balloon open and sensitive to decreases as well as increases in pressure, and helped to keep the cardial balloon in proper position. The sponge rubber was fashioned with sharp scissors, was 3 cm long, 1 cm in diameter, and tapered slightly at both ends. The catheter occupied completely the long axis of the balloon. After insertion it was tied in place at the esophageal fistula and was attached to a sensitive Becker tambour. This caused no discomfort to the dog and did not initiate secondary esophageal peristalsis after the first 2 or 3 insertions in the training period. A 4 cm condom balloon was placed over soft rubber tubing, tied in place and attached to a mercury manometer for recording motility in the loop of jejunum brought to the surface in the first operation.

In making a recording, the cardial balloon was first passed through the esophageal fistula and down the esophagus until it met the moderate resistance of the cardia. About 1 cm more of catheter was allowed to enter the fistula and then the catheter was marked at its point of emergence from the fistula and tied in place. About 3 cc of air were introduced through a Y connection into the tubing connecting the cardial balloon with the Becker tambour. Next the upper esophageal balloon was introduced and tied in position 4 or 5 cm below the fistula and water admitted to the recording system through a Y connection until the mercury manometer was at zero pressure. This same Y connection was used later for introducing 10 cc of water into the balloon to initiate a peristaltic wave. The third balloon was placed in the jejunal loop and 3 to 5 cc of water introduced depending upon the level of motility of the loop. A Y connection in this system allowed for increasing the intraluminal pressure in the jejunum to determine the effect of this on the cardia.

Results. When the dog swallowed spontaneously, or when 10 cc of water were injected fairly rapidly into the upper esophageal balloon and then withdrawn, the cardia relaxed in from 1 to 4 seconds while the peristaltic wave was still high in the esophagus. The maximum relaxation was reached in 5 to 10 seconds or at about the time that the peristaltic wave had reached the cardia. These results are similar to those of Zeller and Burget.[1] Following relaxation, there was a rise in the tonus of the cardia and then a return to normal.

Rhythmic contractions of the jejunum were of large amplitude and were without superimposed periodic increases in pressure in one of the 4 dogs. The others exhibited the more typical variations in amplitude, but at no time was there observed a relaxation of the cardia in response to these spontaneous increases in jejunal pressure. On 2 or 3 occasions, there was noted relaxation of the cardia similar to that produced by intestinal distention, as described below, but without any apparent reason.

It was found that the tonus of the cardia is comparatively insensitive to pressure changes in the jejunum. However, it was demonstrated that repeated, moderate or severe distention of the jejunum may elicit relaxation of the cardia. After recording several typical responses of the cardia to stimulation of the esophagus by distention of the upper balloon, the intestinal balloon was distended sharply with from 4 to 7 cc of water. The amount of water used depended upon the response of the dog, an attempt being made to avoid producing evidence of pain. The pressure was maintained for from one to 3 seconds and then withdrawn. Variations of this procedure were tried in which pressure was maintained for 10 seconds and again in which pressure was alternately raised and lowered in quick succession 4 to 5 times. Neither of these latter 2 variations seemed to alter the result obtained by the first method. The amount of water necessary to elicit relaxation of the cardia varied from dog to dog and from time to time in the same animal. The only obvious factor influencing this amount was the original pressure within the jejunal balloon. When this pressure was high, the volume necessary to elicit relaxation of the cardia was materially reduced. Thus the original tonus of the segment of intestine determines

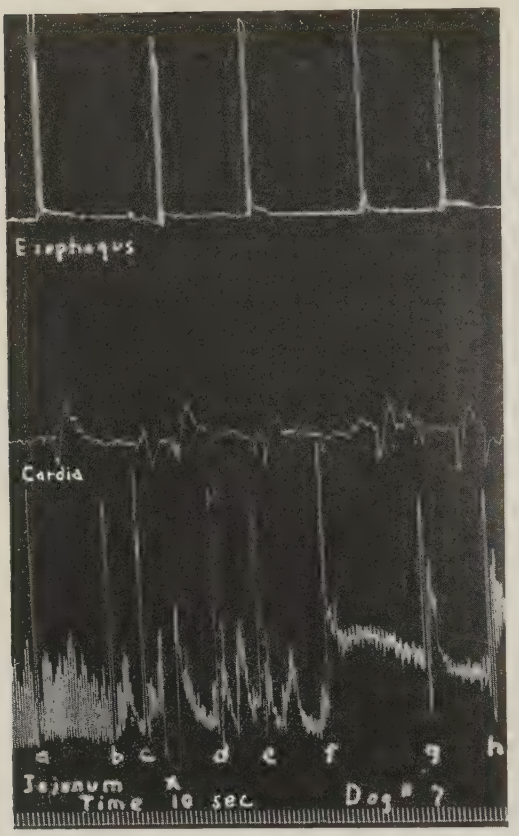

Fig. 1.

Each of the 5 upstrokes on the upper record shows a brief period of pressure produced by introducing 10 cc of water into the balloon in the esophagus. In each case a typical relaxation-contraction pattern was elicited in the cardia. The balloon in the jejunal segment was briefly distended with 7 cc of water at a, b, c, d, e. At f 7 cc were injected and 2 cc removed. At g and h 4 cc were injected and removed. At x the balloon slipped out of the jejunal fistula and was reinserted.

to some extent the sensitivity of this reflex. It usually was found that relaxation of the cardia would not occur with the first 2 to 7 distentions of the intestinal balloon in any experiment, but subsequent distentions with the same volume of water would produce first only slight or questionable decrease in tonus and then unmistakable relaxation of the cardia. Once the correct amount of distention had been determined and the loop had been "sensitized" by preliminary distentions, the relaxation of the cardia could be elicited nearly every time.

The maximum decrease in tonus of the cardia as a result of intestinal distention was usually at least half as great as that obtained as a part of the act of deglutition, and it often approached the amplitude of the latter. Typically, the relaxation occurred in from 2 to 5 seconds, was maximal in 3 to 7 seconds more, and the tonus was back to normal in 15 to 30 seconds. In most cases the relaxation was not followed by a marked rise above the normal tonus level such as that seen in the complexes resulting from spontaneous swallowing or from esophageal distention. The differences in the responses of the cardia to esophageal and to jejunal distention are illustrated in Fig. 1.

No evidence of discomfort or nausea was noted in the dogs at any time as a result of simple distention of the intestinal balloon with small amounts of water and at low pressures. When the pressure had been increased by retaining 4 or 5 cc of water in the balloon, subsequent distentions resulted in a stretching movement of the animal which distorted the record due to a sharp upward deflection of the lever from the cardial balloon. With further increases of pressure up to 80 or 90 mm of mercury the dogs showed evidences of nausea, such as salivation and licking movements, and would eventually vomit if this pressure were maintained. Prolonged relaxation of the cardia of about 60 seconds duration was produced preceding and accompanying signs of nausea in one animal in which the intestinal balloon was distended to the extent of 60 mm of mercury and again to 80 mm of mercury.

The possibility that intestinal distention may alter the motility of the cardia was studied by a second method. The pressure in the intestinal balloon was increased in steps of 10 to 30 mm of mercury by the introduction of 3 to 5 cc of water at a time. Between each increase in pressure the upper esophageal balloon was inflated 2 or more times, initiating a swallow reflex. In nearly all cases the cardia responded with a typical relaxation-contraction pattern upon inflation of the esophageal balloon even with pressures within the jejunal balloon as high as 80 to 90 mm of mercury.

Comment. Intestinal distention is capable

of provoking vomiting, and relaxation of the cardia has been assumed to precede or accompany the vomiting act. These experiments indicate that the cardia is not easily influenced by alterations in pressure in a short isolated jejunal segment. However, relaxation of the cardia may occur in the dog with a moderate, localized jejunal distention without accompanying signs of nausea or other distress. The brief time interval between jejunal distention and relaxation of the cardia necessitates the assumption that this is a reflex. The pathways for the reflex have not been studied. However, the surgical procedure for preparing the jejunal fistula eliminates the possibility of this being an intrinsic reflex. If intrinsic pathways were present it is possible that they might contribute to the effect on the cardia.

It is possible, as in the case of the intestino-intestinal inhibitory reflex,[2] that this reflex could be elicited with less pressure if a longer segment of jejunum were distended. It is also quite possible that the cardia may be influenced more readily by duodenal than by jejunal distention.

Summary and Conclusions. Experiments were undertaken in unanesthetized dogs to study the effect of distention of a segment of jejunum, in the form of a Thiry fistula, upon the tonus of the cardia and upon the relaxation of the cardia resulting from swallowing or from esophageal distention. It was found that the cardia is not easily influenced by changes in jejunal pressure. However, repeated moderate distention of a short jejunal segment may produce a decrease in tonus of the cardia without evidences of nausea or other distress. Secondly, it was found that distention of a jejunal segment does not alter the relaxation-contraction pattern of the cardia which is produced by the swallowing act or by esophageal distention.

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15401

Isolation of an Antibiotic Agent Derived from a *Phycomyces* Active *in vitro* Against *Trypanosoma equiperdum.**†

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The more important antibiotic agents obtained from microorganisms have attracted attention largely because of their antibacterial properties. Any concomitant activity against fungi, actinomycetes, protozoa, or viruses has usually been observed only subsequently, and coincidental to the selective antibacterial effects. This is true, for example, of the antifungal action of streptothricin,[1] the inactivation of certain bacteriophages by streptomycin,[2] and the parasiticidal activity of tyrothricin.[3] Actinomycin and streptothricin prolonged the lives of mice infected with *Trypanosoma equiperdum.*[4] *Trichomonas vaginalis* has been killed *in vitro* by gram-

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† The investigations on the isolation of the organism and isolation of the active substance, carried out at the New Jersey Station, were aided by a grant made by the Commonwealth Fund of New York.

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TABLE I.
Trypanocidal Action of Active Fungus Filtrates.

Organism	Medium	Filtrate "trypanocidal" in dilution*
Dematium sp.	Czapek-Dox	>1:2 but <1:8
Penicillium luteum-purpurogenum	Trypanosome†	>1:2 but <1:8
Penicillium sp. No. 2	Czapek-Dox	>1:8
Penicillium sp. No. 10	Corn steep liquor	>1:2 but <1:8
Phycomyces sp.	Glucose-peptone	>1:2 but <1:8

* Organisms immobilized in 90-180 min at room temperature.
† *T. equiperdum* cells as the sole source of nitrogen.

icidin.[3] More recently, tyrothricin administered in a 95% alcohol solution has been reported to be curative against sporozoite- or blood-induced infections of *Plasmodium gallinaceum* in chickens.[5] Only a few agents, notably gliotoxin[6] and the *B. simplex*[7] factor, were studied largely because of their antifungal properties.

This report presents the results of a study begun in December 1943, with the purpose of isolating an antibiotic agent active against *Trypanosoma equiperdum*.

Despite the controversial status of specific soil enrichment as a technic for the isolation of antagonistic microorganisms,[8] it was decided to use this method of approach. Glass tumblers were filled about two-thirds full with a fertile field soil which had been sifted to remove coarser material, and which approximated a sandy loam in texture. Its pH was approximately 6.5. Throughout the experiments, the tumblers were incubated at 28°C, the moisture content of the soil maintained at a favorable level, and frequent mixing facilitated aeration.

Eleven enrichments of *T. equiperdum* were added to the soil, at irregular intervals, over a 3-month period. Each treatment consisted of about 1.0 g or less of trypanosomes concentrated by differential centrifugation from rat or mouse blood obtained as near the height of infection as possible. Since it was necessary to transport the protozoa from one laboratory to another, the sedimented organisms were frozen with dry ice. Consequently, only dead trypanosomes were added to the soil, and to the selective medium subsequently used for the isolation of antagonistic microorganisms.

After the fourth and again after the last enrichment, the soil was plated out on a medium consisting of 1.5% washed agar, 1% glucose, 0.05% K_2HPO_4, 0.05% KH_2PO_4, and distilled water. A suspension of sedimented *T. equiperdum* was added to this medium to supply nitrogen, in an amount sufficient to produce a definite turbidity. Plates prepared with this medium allowed the development of fungi and of a few bacteria. These organisms were isolated, and grown in different liquid media at 28°C for varying periods of time, after which the filtrates were tested for trypanocidal action *in vitro*.

When tested by an *in vitro* assay in which trypanosomes were exposed at room temperature to varying dilutions for 90-180 minutes, the non-sterile filtrates of 5 fungi were found to exhibit limited antiprotozoan activities (Table I) which could not be attributed to unfavorable pH. However, when the sterilized culture filtrates were left in contact with the organisms for 18 hours, the filtrate of one fungus, which was later found to be a *Phycomyces*, proved to be trypanocidal at much higher dilution, which varied markedly in individual experiments. Further studies were carried out with this organism, which appeared to belong either to the genus *Phytophthora* or *Pythium*.

The active factor was produced by this phycomyces in a glucose-peptone broth as

[5] Taliaferro, L. G., Coulston, F., and Silverman, M., *J. Inf. Dis.*, 1944, **75**, 179.

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[7] Allen, M.C., and Haenseler, C. M., *Phytopath.*, 1935, **25**, 244.

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TABLE II.
Trypanocidal and Antibacterial Activity *In Vitro* of Known Antibiotic Agents in Comparison with Material Derived from *Phycomyces* sp.

Antibiotic*	Highest dilution of solution which inhibited		
	T. equiperdum	*E. coli*	*B. subtilis*
Chaetomin	1:64		1:10,000
Clavacin	1:16,600		1:100,000
Fumigacin	1:500		1:10,000
Streptomycin	<1:200	1:200	
Streptothricin	1:2	1:1,000	
Second antibiotic produced by *S. griseus*†	1:300		1:1,000,000
Phycomyces factor	1:100-1:1,000	<100	<100

* Crude concentrated preparations.

† The second antibiotic factor obtained from *S. griseus* is an alcohol soluble agent extracted with ether from the mycelium of the organism.

well as in a synthetic glucose-asparagin medium. It could be adsorbed on Norit and eluted with organic solvents. The agent was alcohol-soluble and water-soluble. Higher concentrations were obtained by extraction of the pellicles from 10- or 12-day-old cultures with pyridine or benzene. By this procedure extracts were obtained which varied widely in activity, but usually immobilized trypanosomes in 1:100-1:1000 dilution. The latter was approximately 500 times more active than the untreated culture filtrate. The phycomyces factor may, therefore, be a lipid, a small amount of which may saturate the aqueous culture fluid whereas most of the substance is retained within the mycelium.

Solutions of the phycomyces factor possessed slight, if any, action against *E. coli, B. subtilis, B. mycoides,* and *S. lutea.* A comparison of the trypanocidal and antibacterial action of this agent and some well-known antibiotics is presented in Table II. The latter were crude preparations, their activity being indicated by their effect upon specific bacteria. The selective effect of the phycomyces factor upon the protozoan is of interest.

In white mice, the toxicity of an alcoholic concentrate of the agent was no greater than that of the alcohol. Similarly, the substance exerted no demonstrable therapeutic action in mice infected with *T. equiperdum* and treated with maximal tolerated doses of the alcoholic solution. The absence of toxicity and efficacy indicate that the compound was probably present in low concentration.

Summary. Several soil fungi have been found to produce filtrates active against *Trypanosoma equiperdum.* From a *Phycomyces* sp. a lipid-like substance was isolated which immobilized the trypanosome *in vitro* but exerted no protective action against the experimental infection in mice.

No inhibitive agent has heretofore been obtained from this group of fungi. The selective action of the phycomyces factor against the trypanosome, but not against bacteria, is of particular interest.

15402

Hypertensinase Activity of an L-Aminoacid Oxidase Preparation and of the Venom of *Bothrops neuwidii.*

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There is a certain degree of agreement concerning the possible hydrolytic nature of hypertensinase as an enzyme. Nevertheless its inactivation by tyrosinase (Schroeder and Adams,[1] Croxatto *et al.*[2]), by oxidized cytochrome, and by the oxidizing effect of certain reagents (Cruz Coke[3]) suggest that anoxemia might impair the ability of tissues to destroy the vasoconstrictor properties of hypertensin, as in the case of kidney ischemia. Furthermore, Raska[4] reported that oxygen consumption and the oxidative deamination of amino acids by slices and extracts of kidneys from hypertensive dogs are markedly decreased as compared to kidneys from normal animals.

The fact that the kidney is one of the richest sources of hypertensinase and also has a high *d*- and *l*-amino-acid oxidase activity led us to study the action of preparations of the latter enzyme on hypertensin. As aminopolypeptidase hydrolyzes hypertensin, (Croxatto and Croxatto[5]) this substance must contain free amino groups. Blanchard *et al.*[6] were able to separate *l*-amino-acid oxidase from rat kidney and a similar enzyme was recently shown to be present in snake venom by Zeller and Maritz.[7] Croxatto and Croxatto[8] have shown a high hypertensinase activity in several of these venoms. We attribute this activity to proteolytic enzymes, in spite of the fact that the activity is higher than that of any single crystallized proteolytic enzyme.

In the following experiments, we have used an *l*-amino acid oxidase preparation obtained from rat kidneys by the method of Blanchard *et al.* and the dry venom of a South American snake, *Bothrops neuwidii.*‡ Hypertensin was prepared by the method of Braun Menendez *et al.*[9]

Experiments. Kidney l-amino-acid oxidase. A series of tubes containing 10-20 U of hypertensin per ml, 0.2 M acetate or phosphate buffer (pH 5, 7.4, and 10.4) and different concentrations of *l*-amino-acid oxidase were incubated at 37.5° with aseptic precautions. Tubes containing all the other elements but lacking the enzyme preparation were treated in the same way and served as controls. The rate of hypertensin inactivation was determined by the method of Fasciolo *et al.*[10] by injecting aliquots of the incubated mixture into the femoral vein of an anesthetized cat ("Dial") and comparing the effect on blood pressure with that of the control. The sample used for determination was previously deproteinized by boiling in a water bath and centrifuging to separate the precipitate; the

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‡ A high *l*-amino-acid oxidase activity in this venom was recently found by D. Green (personal communication).

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TABLE I.
Hypertensinase Activity of *l*-aminoxidase Extract.

Enzyme ml*	Time of incubation min	pH	Oxygen†	Inhibitor	Conc. M	Hypertensin inactivation %
0.02	400	7.4	+	—		60
0.02	400	,,	—	—		60
0.05	60	,,	+	—		50
0.05	60	,,	—	—		50
0.05	180	,,	+	—		100
0.1	100	,,	+	—		80
0.1	100	,,	—	—		80
0.1	60	5.4	+	—		70
0.1	60	10.5	+	—		80
0.1	60	7.4	+	—		100
0.02	400	7.4	+	Salicylic acid	0.4	60
0.05	60	,,	—	Ammonium sulphate	0.25	50
0.05	180	,,	+	,,	,,	100
0.1	100	,,	+	,,	,,	80
0.05	180	,,	+	*l*-leucine	0.1	100
0.05	180	,,	—	,,	,,	100

* Three units of hypertensin were incubated at 37.5°C with the amount of enzyme indicated in this column.

† (+) Indicates presence of oxygen and (—) absence of oxygen in the mixture.

TABLE II.
Hypertensinase Effect of *Bothrops neuwidii* Venom.

Dried venom µg*	Time min	Oxygen†	Inhibitor	Conc. M	Hypertensin inactivation %
1	90	+	—		100
1	90	—	—		100
1	60	—	—		80
1	60	+	—		80
2.5	15	—	—		40
2.5	15	+	—		40
2.5	20	+	—		40
2.5	30	+	—		60
1	60	—	Salicylic acid	0.4	80
1	60	+	,,	,,	80
2.5	30	+	,,	,,	60
2.5	30	—	,,	,,	60
2.5	20	+	4 nitrotoluen sulf.	0.15	40
2.5	20	—	,,	,,	40

* Three units of hypertensin were incubated at 37.5°C with the amount of the venom indicated in this column.

† (+) Indicates presence of oxygen and (—) absence of oxygen in the mixture.

same procedure was applied to the control. Additional experiments were performed in Thunberg tubes, one series in anaerobic conditions obtained by high vacuum and another in pure oxygen.

Table I shows that hypertensin was inactivated by *l*-amino-acid oxidase preparation and that a complete inactivation of 3 U of hypertensin was achieved by 0.05 cc in 3 hours at pH of 7.4. A lower activity was observed at pH 5.4 and 10.4.

The rate of hypertensin inactivation was the same under aerobic and anaerobic conditions.

The same table shows that 0.25 M ammonium sulfate does not inhibit the inactivating action on hypertensin, contrary to the effect shown on amino acid deamination by Blanchard *et al.* No competitive effect of 0.1 M *l*-leucine was observed; inactivation went on at the same rate as in its absence.

Bothrops neuwidii venom. Applying the technic already described, we studied the effect of this venom on hypertensin. Amounts

of dried venom ranging from 1 to 5 μg were added to 3 U of hypertensin in a medium buffered to pH 7.4 where the venom shows maximum hypertensinase activity.

The results obtained are summarized in Table II. There was no difference in the rate of inactivation whether oxygen was present or absent. The effect of 4-nitrotoluene sulphonic acid and of salicylic acid was studied by addition of 10 mg of the first compound or 20 mg of the second (adjusted to pH 7.4) for every 3 U of hypertensin. Since there was no change in the course of the inactivation, this process is probably not due to the action of the *l*-amino-acid oxidase, as Zeller *et al.*[7] have reported inhibition of this enzyme by these 2 substances.

There are further differences between the inactivation of hypertensin and the deamination of amino acids by *l*-amino-acid oxidase extract. The hypertensinase activity is not affected by absence of oxygen or presence of ammonium sulfate. The inactivation of hypertensin is at a maximum at pH 7.5 and does not decrease sharply when the pH is decreased or increased. According to Blanchard *et al.* the rate of oxygen uptake and ammonia production from amino acids has a pH maximum about 10. Above and below this pH the velocity of oxidation declines sharply, being negligible at pH 7.

Conclusion. The hypertensinase activity shown in the same preparation of *l*-amino-acid oxidase used by Blanchard *et al.* may be attributed to another enzyme, associated as an impurity. The results obtained with the venom of *Bothrops neuwidii* support our previous view that the hypertensinase activity of this venom is due to the presence of a proteolytic agent, and do not support the hypothesis that the *l*-amino-acid oxidase participates in the inactivation of hypertensin by the same venom.

I am indebted to Dr. D. E. Green for his generous supply of *l*-amino-acid oxidase and to Prof. O. Krayer for his kindness in putting the facilities of his department at my disposal.

15403

Effect of Steroid Sex Hormones on Distribution of Alkaline Phosphatase in Uterus of Mouse.*†

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The distribution of alkaline phosphatase in the uterus during the reproductive cycle and pregnancy has been described in several species[1–3] following the development by Gomori[1,4] of methods for demonstrating the presence of this enzyme in tissue sections. It is generally agreed that alkaline phosphatase may be found in any of the component tissues of the endometrium, the exact distribution varying with the physiological state and the species studied. Although the specific function of phosphatase in the physiology of reproduction is not clear, there is reason to believe that

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† The progesterone (Progestin) used in this work was supplied through the courtesy of Roche-Organon, Inc., Nutley, N. J. The estradiol benzoate and testosterone propionate were made available by CIBA Pharmaceutical Products, Inc., Summit, N. J.

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TABLE I.
Amount and Distribution of Alkaline Phosphatase in the Uteri of Ovariectomized Mice Treated Various Steroid Sex Hormones.

Group	Treatment (dose/day for 3 days)	No. of animals	Myometrium		Endometrium		
			Outer long.	Inner circ.	Uterine epith.	Glands	Stroma
1.	Untreated	6	+++	+	±	±	0
2.	1.0 μ estradiol benzoate	5	+++	++	+++	+++	0
3.	1 mg progesterone	5	+++	0	±	±	0
4.	1.0 μ estradiol benzoate 1 mg progesterone	5	+++	+	+++	+++	0
5.	0.5 mg testosterone propionate	5	+++	±	±	±	0

it plays an integral part. Many phases of the reproductive process have proven to be under the influence of the gonadal hormones. The following experiments were designed to ascertain whether the administration of steroid sex hormones influences the distribution and amount of alkaline phosphatase in the uterus of the mouse.

Experimental. Twenty-six young adult mice of the Swiss albino strain were bilaterally ovariectomized. A control group of 6 animals received no further treatment and was killed 7 to 14 days after operation. The remaining mice were divided into 4 groups of 5 each (Table I). Daily subcutaneous injections of several steroid sex hormones were begun 3 to 14 days after ovariectomy. Crystalline hormones were used; the daily dose was dissolved in 0.05 to 0.1 cc of sesame oil. The animals in the second and third groups received 1.0 μg of estradiol benzoate and 1 mg of progesterone respectively each day. The fourth group were injected daily with both 1.0 μg of estradiol benzoate and 1 mg of progesterone. The last group received 0.5 mg of testosterone propionate daily. Treatment was continued for 3 consecutive days and the animals were killed by illuminating gas 24 hours after the last injection. The animals were autopsied immediately and the uteri were fixed in chilled 80% ethyl alcohol for 24 hours. After dehydrating and imbedding in paraffin, sections were cut 5 micra in thickness. The tissue sections were incubated in a 1% sodium glycerophosphate substrate for 3 hours at 35° to 39°C and treated according to the technic of Gomori.[1,4] This results in a dark deposit of cobalt sulfide at the sites of alkaline phosphatase activity. The sections were counterstained with Ehrlich's hematoxylin and were mounted permanently for subsequent study.

In the untreated ovariectomized mice deposits of cobalt sulfide, indicating phosphatase activity, were dense in the muscle cells of the longitudinal layer of the myometrium and were very light in the circular muscle. (Table I). Occasional light deposits of sulfide were also present in the uterine glands and epithelium. No evidence of cytoplasmic enzyme activity was present in the endometrial stroma. On the other hand, in the animals injected with estrogen there was a marked increase in the enzyme activity in the uterine glands and epithelium and in the circular layer of the myometrium. The sulfide deposits in the cells of the epithelium and glands tended to lie apical to the nuclei, being particularly dense at the free borders of the cells. Frequently deposits were evident in the connective tissue cells immediately adjacent to the glands. The remainder of the stroma exhibited no enzyme activity.

The distribution of phosphatase in the uteri of the animals treated with progesterone and androgen was essentially the same as in the untreated controls. The only consistent exception was the absence of detectable sulfide deposition in the circular muscle in the progesterone-treated group. In the mice receiving estrogen and progesterone concurrently the sites of phosphatase activity appeared to be the same as those described in the animals

receiving estrogen alone.

Discussion. It has been shown clearly in the present experiments that the injection of estrogen into the ovariectomized mouse is followed by a marked increase of alkaline phosphatase in the lumenal epithelium and that of the uterine glands and in the circular layer of muscle. It is also evident that neither progesterone nor androgen have this effect. Progesterone given concurrently with estrogen does not modify the effect of estrogen on uterine phosphatase.

The distribution of alkaline phosphatase in the endometrium of the ovariectomized mouse treated with estrogen is similar to that described during the reproductive cycle of the dog, rabbit and guinea pig[1] and the human.[2] Like the human, however, no widespread enzyme activity is evident in the endometrial stroma. Since the uterine specimens used in these previous studies were not timed as to the stage of the cycle, it is impossible to determine whether the phosphatase distribution could be related to estrogenic activity. In the cases where the endometrial specimen was obtained during advanced pregnancy, it is possible to assume that in some of the species the level of estrogen secretion had been high. In the pregnant rat and guinea pig[3] the distribution is similar to that in our estrogen-treated mice. However, in the human the enzyme is found only in the endothelium of the blood vessels during early pregnancy and later disappears entirely. In the pregnant sow the phosphatase is confined to the endothelial cells and to the endometrial stroma. It is obvious that no generalization can be made from such divergent observations until further data on the hormone-enzyme interrelation are available in these species.

The presence of alkaline phosphatase in the myometrium and the variations in its distribution after hormone treatment present problems which await explanation. Earlier studies have indicated that the myometrium of the human uterus does not contain phosphatase during the menstrual cycle.[2]

The significance of phosphatase in metabolism is due in part to its participation in the phosphorylation and dephosphorylation of many biologically important compounds. It is interesting to note that estrogen causes an increase in the glycogen content of the rat uterus.[5] It is possible that there is a relationship between alkaline phosphatase and the appearance of glycogen under these circumstances. In addition, estrogen causes the disappearance of the lipids in the uterine glands and epithelium of the rat,[6] an event which might also be related to phosphatase activity.

Summary. The distribution of alkaline phosphatase was studied in the uteri of untreated ovariectomized mice and in castrates injected with 3 steroid sex hormones. In the untreated castrate large amounts of the enzyme are present in the longitudinal muscle and smaller amounts are found in the circular muscle. Occasional small amounts are also present in the uterine glands and epithelium. Injection of estrogen is followed by marked increase of phosphatase in the uterine glands and epithelium and in the circular muscle. Progesterone and androgen do not have this effect. Progesterone given concurrently with estrogen does not alter the enzyme response to estrogen.

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15404

Some Factors Which Decrease Arterial Saturation in Bird Malaria-Ducks Infected with *P. lophurae*.*†

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The failure of oxygen transport during the terminal stage of *P. lophurae* infection in the duck has been suggested by Rigdon and Rostorfer[1] to be the cause of death. The anemic anoxia is so severe that the oxygen capacity of the blood is only 18 to 19% of the normal. Accompanying this low oxygen capacity there is a marked decrease in percentage of saturation of the blood *in vitro*. Wong[2] was able to demonstrate a decrease in arterial saturation *in vivo* during malarial paroxysms in neurosyphilitic patients undergoing malarial fever therapy. Arterial saturations as low as 70% were noted during the peak of the fever. In discussing the cause of this failure of arterial saturation Wong states, "the presence of some factor which was inhibiting full oxygenation of the blood hemoglobin might be proposed as an explanation of this phenomenon."

This "factor" which Wong suggests might conceivably be either chemical in nature and peculiar to malaria, or it might be a combined physiological action such as the combined effect of temperature increase, acidosis or alkalosis. Furthermore, the mechanics of the circulation and respiration such as the speed of circulation and fall of alveolar partial pressure of oxygen might be contributory factors. In other words this same phenomenon might be observed generally in severe febrile conditions. On the other hand, if some factor prevents oxygen uptake by the hemoglobin in *P. vivax* infection in the human, one might expect to find a similar factor in the blood in other types of malaria, for example, *P. lophurae* infections in the duck. Accordingly, blood from *P. lophurae* infected ducks was investigated *in vitro* with the aim of demonstrating the presence of some factor inhibiting oxygenation of the blood. The relationship of oxygen saturation to pH, pK, CO_2 combining power, and temperature in malarial-infected duck blood is reported and discussed in this paper.

Methods and Materials. In this study *P. lophurae* obtained from highly parasitized donor birds was used as the inoculum to infect young white Pekin ducks from 15 to 20 days old. The parasitemia was followed by counting the number of parasitized cells in 500 red cells from smears stained with a combination of Giemsa's and Wright's stains. Standard methods were used in making the total red cell counts. Young erythrocytes were differentiated from adult red cells by the darker staining of the cytoplasm and rounder nucleus. Blood samples for analytical purposes were obtained by heart puncture from 2 or 3 birds in the same stage of infection. These samples were pooled. Coagulation was prevented by using 1 ml of a 1% solution of the sodium salt of heparin for each 9 ml of blood. All cell counts and gas analyses were obtained from these pooled samples. Equilibration and gas analyses were carried out in the manner described by Rostorfer and Rigdon.[3] Normal and malarial duck blood was equilibrated at 38°C and 42°C and the oxygen dissociation curves at these temperatures were calculated by the use of the formula $= Y = 100\ kX^n/1 + kX^n$.

* This work was supported by a grant from the John and Mary R. Markle Foundation to the Department of Pathology. The anti-coagulant used in these experiments was "Lequaemin" supplied by Roche-Organon, Inc., Nutley, N. J.

† Research paper 585 Journal Series, University of Arkansas.

1 Rigdon, R. H., and Rostorfer, H. H., *Nat. J. Mal. Soc.*, in press.

2 Wong, Yan Tim, *Science*, 1945, **102**, 278.

3 Rostorfer, H. H., and Rigdon, R. H., *Am. J. Physiol.*, 1946, **146**, 222.

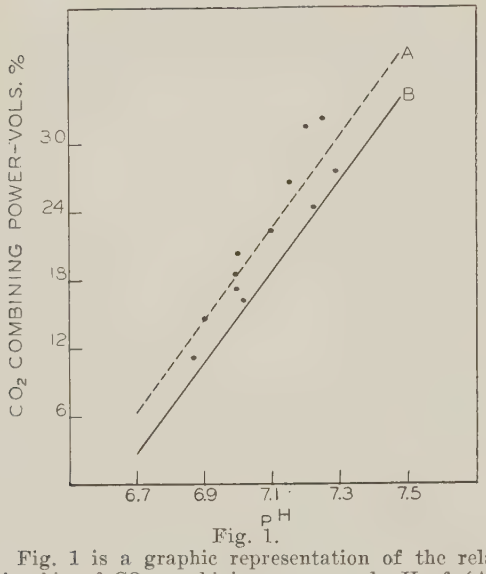

Fig. 1.

Fig. 1 is a graphic representation of the relationship of CO_2 combining power and pH of (A) normal duck blood and (B) blood of malarial-infected ducks during different stages of the infection. The normal relationship (line B) is calculated by the use of the normal pK of 6.20. The pK for line A is the average pK for malarial blood, namely 6.05.

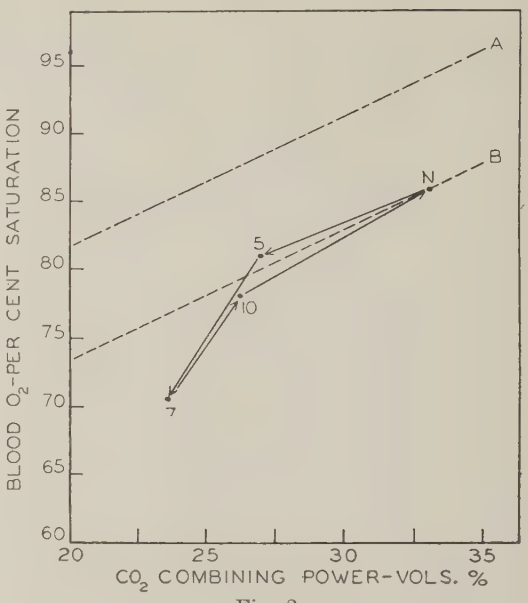

Fig. 2.

Fig. 2 is a graphic representation of the relationship of the percentage of oxygen saturation at an O_2 tension of 83 mm Hg with the CO_2 combining power for (N) normal duck blood, (5) fifth day after inoculation, (7) seventh day after inoculation, and (10) tenth day after inoculation. Line A is the rate of fall in saturation with increasing CO_2 tension in the equilibration gases. Line B is the theoretical rate of decreasing saturation with decreasing CO_2 combining power.

The constant k and the exponent n were calculated from the data obtained by equilibrating samples at 52 and 83 mm Hg of oxygen tension. The pH was determined on samples equilibrated at 31 mm Hg of CO_2 tension by the use of the glass electrode. The pK was calculated by the use of the formula $pH = pK + \log BHCO_3/\text{Free } CO_2$.

Results. A severe anemia develops during the course of *P. lophurae* infection in the duck. In the terminal stage of the infection the blood may have only 15% of normal oxygen capacity. As the anemia progresses there is a decrease in pH which is definitely correlated with the degree of anemia, at least during the terminal stages of the disease. pH values of heparinized blood taken during the 5th and 6th day after inoculation have been recorded as low as 6.89. A fall in pH from the normal of 7.40 to 7.25 was noted as early as the third day.

The CO_2 combining power of the whole blood out of normal young ducks, which was heparinized as described and equilibrated with 31 mm Hg of CO_2 tension, was found to be between 33 and 34 volumes %. There is a gradual fall in CO_2 combining power during the course of the malarial infection. Values as low as 14 volumes % have been observed. This shift of CO_2 combining power is accompanied by a corresponding fall in pH. The relationship of CO_2 combining power and pH is shown in Fig. 1.

The pK of normal blood from young ducks is approximately 6.2. However, during the course of malarial infection the pK falls to values as low as 6.05. In Fig. 1, line B represents the relationship between CO_2 combining power, pH and pK for the blood of normal young ducks. Line A is the average relationship of these 3 values during the course of infection. It can be seen in Fig. 1 that the pH is lower at the same level of CO_2 combining power in the malarial blood in comparison to the normal blood.

The decrease in blood oxygen saturation which accompanies the decrease in CO_2 combining power is shown in Fig. 2. Line A is

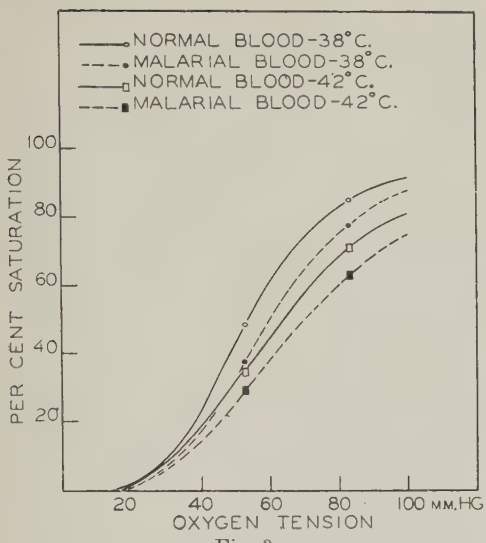

Fig. 3.

Fig. 3 is the graphic representation of the effect of increasing temperature on normal and malarial duck blood.

the normal fall in oxygen saturation which results from increasing the tension of CO_2 in the equilibrating gas. Line B is the normal fall in oxygen saturation with decreasing CO_2 combining power of the blood. According to Adair[4] and Barcroft[5] there is a linear relationship between O_2 uptake with CO_2 combining power and pH which holds until the lower levels of pH are reached when the percentage of O_2 saturation fails to continue to fall with further decrease in pH. It will be noted that malarial blood bears out this relationship until the 7th day of the infection. At this time there is a deviation from the linear relationship. This deviation is in the opposite direction from that which normally occurs at lower pH values. However, if the birds recover from the infection the normal linear relationship is restored by the 10th day.

The one discernible difference in the malarial blood between the 5th and 7th day of infection lies in the great increase in young red cells by the 7th day. At this time and later, if the bird survives, the young cells may reach numbers of between 200 and 400 per 500 red cells. These cells have been shown by Rostorfer and Rigdon[6] to have hemoglobin which is apparently more susceptible to the CO_2 effect, to carry less hemoglobin per cell, and to be less effective as O_2 carriers. By the 10th day after inoculation most of the young red cells have disappeared from the blood stream, and by this time the original linear relation between CO_2 combining power and O_2 saturation has been restored.

The effects of the acidosis and temperature upon malarial blood in relation to the same effects on normal duck blood are shown in Fig. 3. The combined effects of a rise of 4°C in temperature and the acidosis cause the dissociation curves to markedly shift to the right. The malarial blood at 42°C has only approximately 70% of the oxygen combining capacity of normal duck blood at 100 mm Hg of O_2 tension.

In order to demonstrate the effect of any substance or "factor" in the plasma of malarial blood which might affect the cells and decrease oxygen uptake, samples, of 10 ml of heparinized malarial and normal duck blood, were centrifuged and the plasma was drawn off by careful aspiration. The plasma of malarial blood was placed on normal cells and the normal plasma was placed on malarial cells and carefully brought up to the original volumes. These samples were equilibrated with air and with 83 mm Hg O_2 tension and 31 mm Hg CO_2 tension and compared with the data obtained on the original bloods. No difference in O_2 capacities was noted between the original malarial blood and the blood which was composed of malarial cells and normal plasma. Nor was there any demonstrable difference between the O_2 capacities of the original normal blood and that for normal cells and malarial plasma. There was a fall, however, in percentage of saturation in the case of normal cells and malarial plasma equilibrated at 83 mm Hg of O_2 tension and 31 mm Hg of CO_2 tension. There was also a fall in CO_2 combining power in this mixture of malarial plasma and normal cells

[4] Adair, G. S., *J. Biol. Chem.*, 1925, **63**, 529.

[5] Barcroft, J., Camis, M., Mathison, C. G., Roberts, F., and Ryffel, J. H., *Phil. Trans. London*, Series B, 1914-15, ccvi, 69.

[6] Rostorfer, H. H., and Rigdon, R. H., *Am. J. Clin. Path.*, in press.

which seems sufficient to account for the decreased percentage of saturation. There seemed to be no decrease in oxygen uptake by the normal cells in the presence of malarial plasma; nor were the malarial cells benefitted by the normal plasma excepting the increase in saturations resulting from the rise in CO_2 combining power due to the presence of normal plasma.

Discussion. During the course of malarial infection in the duck an anemia develops which is accompanied by a fall in pH, a marked decrease in CO_2 combining power and a decrease in pK. There is also a shift of the oxygen dissociation curve to the right and a decrease in saturation of the blood *in vitro*. This decrease in saturation bears a linear relationship with the pH and the CO_2 combining power with the exceptional case of the blood taken from ducks on the 6th and 7th day of the infection. During this time young red cells make up from 60 to 80% of the total number of cells present. These cells have been demonstrated to have an abnormal behavior with respect of O_2 capacity, acid base binding power and dissociation curve. It is the authors' opinion that the young cells present on the 6th and 7th days after inoculation are responsible for the abnormal decrease in O_2 saturation with the decreased CO_2 combining power which occurs at this time.

When the effect of temperature is superimposed on the effect of acidosis the percentage of saturation is greatly lowered at the higher oxygen tensions of the equilibrating gas. There seems to be no need for the assumption of a "factor," such as suggested by Wong, which prevents the O_2 saturation of blood other than the decreased CO_2 combining power, the fall in pH, the presence of young cells and increased temperature in the case of *P. lophurae* infected duck blood to account for the decrease in oxygen saturation of the blood *in vitro*.

It should be pointed out here that Cullen *et al.*[7] have investigated arterial oxygen saturation in patients whose body temperatures have been raised in the fever box and have observed decreases in arterial saturation down to 70% of the normal value. It should be noted also that the data obtained by Wong are correlated absolutely with the degree of fever. It is quite possible that the decreased arterial saturation in severe febrile condition has a physiological basis. Various investigators are not in agreement as to the mechanisms involved in the cause of decreased O_2 saturations in febrile conditions but the phenomenon does not seem to be peculiar to the fever of malarial infection.

Summary. Data have been presented which indicate that the decreased arterial saturation, *in vitro*, of the blood of malarial-infected ducks may be caused by the state of acidosis and the presence of great numbers of young red cells during the terminal stage of *P. lophurae* infection. The combined effect of increased temperature and acidosis on the *in vitro* oxygen saturation is sufficient to reduce the percentage of saturation to 70%. No evidence was obtained for the existence of a "factor" in malarial duck blood which would prevent the saturation of the hemoglobin of normal red cells obtained from uninfected birds.

[7] Cullen, S. C., Weir, E. F., and Cook, E., *Anesthesiology*, 1942, **3**, 123.

15405

Effects of Reduced Caloric Intake on Leucocyte Count of the Rat.*

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The purpose of the present communication is to report the effects of reduced caloric intake on the leucocyte count of the rat. It

* The subject matter of this paper has been undertaken in cooperation with the Quartermaster Corps Committee on Food Research.

TABLE I.
Effects of Reduced Caloric Intake on the Body Weight, Hemoglobin, and Total Erythrocytes of the Rat.

Exp. group	No. of animals	Initial body wt	Body wt on 56th day	Avg R.B.C. (in millions)	Avg Hb mg/100 cc
Diet A—6 g	6	171	125	9.0 (8.3-10.4)	13.9
" B—6 "	6	167	128	8.5 (8.2- 9.1)	14.3
" A—9 "	6	174	172	8.6 (7.1- 9.9)	13.8
" B—9 "	6	168	188	9.8 (9.3-10.5)	14.6
" A—*ad lib**	6	165	173	8.8 (8.2- 9.4)	14.3
" B—" "*	6	171	208	9.6 (9.2-11.4)	14.6

* Average food consumption per day for the 8-week period in the *ad lib* series was 10.9 g on diet A and 11.9 g on diet B.

TABLE II.
Leucocyte Count of Rats Fed a Reduced Caloric Intake.

Group	Total leucocyte count		Granulocytes	
	Avg*	Range	%*	Total*
Diet A—6 g	7,400± 540	(5,800- 9,800)	11.7±1.5	866±111
" A—9 "	10,340± 860	(8,000-13,200)	9.2±0.5	952± 52
" A—*ad lib*	13,350± 650	(11,000-15,800)	15.0±2.5	2025±338
" B—6 g	8,100± 600	(6,200- 9,800)	36.8±2.4	2980±194
" B—9 "	11,000± 960	(7,800-14,200)	24.8±1.6	2728±176
" B—*ad lib*	15,600± 820	(12,700-18,000)	20.8±3.0	3245±468
Effects of folic acid therapy				
" A—6 g	21,900±3800	(13,800-33,200)	28.2±4.4	6175±964
" A—*ad lib*	13,900± 770	(12,300-16,100)	16.7±3.1	2321±431

* Including standard error of the mean calculated as follows:

$$\sqrt{\frac{\Sigma d^2}{n}} \Big/ \sqrt{n}$$

where "d" is the deviation from the mean and "n" is the number of observations.

has been demonstrated by a number of investigators that diets adequate for apparently normal growth and development cease to be adequate when animals are subjected to various toxins, infection, drugs, lactation and other "stress factors." This inadequacy is reflected in the blood picture to the extent that leucopenia and granulocytopenia develop on rations which support a normal blood picture in the absence of these factors.[1-4] In the present experiment we were interested in determining whether the adjustment to reduced caloric intake would constitute a "stress factor" sufficiently pronounced to manifest itself in changes in the leucocyte count.

Procedure and Results. Female rats of the U.S.C. strain were raised to maturity on a stock ration and were selected for the present experiment at approximately 110 days of age and an average body weight of 169 g (range 151 to 200 g). Two basal rations were employed: diet A and diet B.† Diet A was a

[1] Sebrell, W. H., and Daft, F. S., *Pub. Health Rep.*, 1943, **58**, 1542.

[2] Goldsmith, E. D., Gordon, A. S., Finkelstein, G., and Charipper, H. A., *J. Am. Med. Assn.*, 1944, **125**, 847.

[3] Daft, F. S., Kornberg, A., Ashburn, L. L., and Sebrell, W. H., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 154.

[4] Nelson, M. M., Van Nouhuys, F., and Evans, H. M., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 74.

† Diet A consisted of the following: Vitamin Test Casein 30, Sure's Salt Mixture No. 15 4.5, cottonseed oil 10 and sucrose 55.5. To each kg of the above were added 2 mg thiamine HCl, 4 mg riboflavin, 2 mg pyridoxine HCl, 3 mg calcium pantothenate, 1200 mg choline chloride and 5 mg 2-methyl-napthaquinone. Diet B consisted of Vitamin Test Casein 30, Sure's Salt Mixture No. 1 4.5, cottonseed oil 10, sucrose 45.5 and

purified ration containing the B complex factors in synthetic form; diet B was similar in composition but contained yeast in place of the synthetic B factors. Both rations were administered at 3 levels of caloric intake: (1) 6 g daily, (2) 9 g daily and (3) *ad lib.* Animals were kept in individual cages with screen bottoms to prevent access to feces; body weight was recorded weekly; and food intake was determined daily for the *ad lib* series. Thirty-six rats were employed in the present experiment, consisting of 6 groups of 6 animals each.

After 8 weeks of feeding total and differential white cell counts, hemoglobin determinations and total red cell counts were made on the tail blood of all rats. Differential counts were made on smears stained with Wright's stain, 100 cells on each of 2 slides being employed for each analysis. All blood counts were made in duplicate.

Results are tabulated in Tables I and II. No significant differences in total erythrocytes nor hemoglobin levels were observed in any of the rations at any of the levels fed. Significant reductions in total leucocytes however were observed when animals were fed a reduced caloric intake, the highest value for the 6 g per day series being smaller in every instance than the lowest when these rations were fed *ad lib,* values for the 9 g per day series being intermediate between the 2. Differences were similarly noted in per cent and total granulocytes. On Diet B a marked increase in per cent granulocytes was observed in the 6 g per day series so that total granulocytes per cc of blood were equal to levels present in the *ad lib* series; no such increase occurred with reduced caloric intakes on diet A with the result that total granulocytes in this series were reduced by more than 50%. Inasmuch as diets A and B differed only in their content of yeast, these findings suggest that yeast may contain some factor essential for the maintenance of a normal granulocyte count under conditions of reduced caloric intake.

A number of reports have appeared concerning the curative effects of folic acid on granulocytopenia induced by sulfonamide feeding,[1,6] diet alone,[7] deficiency of pantothenic acid[8] or riboflavin,[9] or administration of thiourea and thyroxin.[3] Accordingly folic acid was administered to animals on diet A on both the 6 g per day and *ad lib* levels. Crystalline folic acid‡ was fed for 4 days at a level of 50 γ daily, and on the 5th day total white cell and differential counts were determined. Results are summarized in Table II. A significant increase both in total leucocytes and granulocytes occurred on the 6 g per day level. Total leucocytes ranged from 13,800 to 33,200 per cc after 4 days of folic acid feeding with an average increase of 300%; while granulocytes averaged 28% (range 2208 to 13,944 per cc). No significant differences in total leucocyte or granulocyte count were observed subsequent to folic acid administration in animals fed *ad lib.*

These findings suggest that the low granulocyte counts observed on diet A when fed at reduced caloric intakes may have been due in part to a deficiency of folic acid, while sufficient amounts of this nutrient were present in yeast to meet body requirements for this factor on diet B under similar experimental conditions. Results indicate further that under conditions of the present experiment caloric restriction on diet A interfered either with the synthesis or utilization of folic acid or increased body requirements for this factor to an amount greater than that being synthesized by intestinal microorganisms. Rats on a reduced caloric intake of diet A thus

yeast (Hi-Ribo-24, Anheuser-Busch, Inc.) 10. To each kg of the above were added 1200 mg choline chloride and 5 mg 2-methyl-napthaquinone. Each rat on diet A or B also received a weekly supplement containing 100 U.S.P. units of vitamin A and 10 U.S.P. units of vitamin D.

[5] Sure, B., *J. Nutrition,* 1941, **22**, 499.

[6] Endicott, K. M., Daft, F. S., and Ott, M., *Arch. Path.,* 1945, **40**, 364.

[7] Kornberg, A., Daft, F. S., and Sebrell, W. H., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 46.

[8] Daft, F. S., Kornberg, A., Ashburn, L. L., and Sebrell, W. H., *Pub. Health Rep.,* 1945, **60**, 1201.

[9] Kornberg, A., Daft, F. S., and Sebrell, W. H., *Arch. Biochem.,* 1945, **8**, 431.

‡ We are indebted to Dr. Stanton M. Hardy of Lederle Laboratories, Pearl River, N. Y., for the folic acid employed in the present experiment.

responded to folic acid administration inasmuch as they were presumably deficient in this factor; whereas animals fed the same ration *ad lib* had sufficient amounts of folic acid available presumably through intestinal synthesis for an optimal effect.

Summary. A significant reduction in total granulocytes was observed in female rats fed a synthetic ration at reduced caloric intake. Such reduction was not observed in animals fed a similar ration *ad lib* or yeast in place of the synthetic B vitamins. The low granulocyte counts were corrected by treatment with folic acid.

15406

Influence of Streptomycin and Promin* on Proliferation of Tubercle Bacilli in the Tissues of Albino Rat.

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It has been shown[1] that streptomycin and promin when administered together, the one intramuscularly and the other orally, to guinea pigs infected with a human strain of tubercle bacilli, exerted a chemotherapeutic action greater than what might have been anticipated from simple summation of effects. No information is as yet available on the mechanisms that might be involved in this synergistic action. The effect might conceivably be a direct one, on the invading microorganism, or it might be an indirect effect on the defense mechanisms of this normally susceptible host.

In the present work experiments were undertaken to study this phenomenon in the albino rat, normally a highly resistant host to tubercle bacillus infection. It was shown in 1926[2] and amply confirmed since then[3-8] that intraperitoneal or intravenous inoculation of a virulent strain of human or bovine tubercle bacilli in the white rat results within a month in progressive proliferation of monocytes, forming aggregates of epithelioid cells, with some multinucleated, and occasionally a few giant cells. These cellular aggregates are found predominantly in the lungs and to a lesser extent in the liver and spleen. Tubercle bacilli multiply at the same time in great numbers in these cells without producing necrosis, caseation, or other serious tissue damage. The whole process appears to present a simple host-parasite symbiosis having little effect on the general health of the animal until much of the normal lung tissue is replaced by the epithelioid masses which ultimately so impair the normal respiratory functions by reducing the available alveolar space that the animal dies of asphyxia.

In an attempt to learn more about the manner of action of streptomycin and promin it was aimed in the present experiments to ascertain the influence of these drugs, when used individually or in combination, on the proliferation of tubercle bacilli in the tissues of the rat, their viability when planted on culture media, and their pathogenicity when inoculated into guinea pigs.

Experimental. Four groups of rats, 12 each, weighing 100 to 150 g, were inoculated intraperitoneally with 5 mg tubercle bacilli, human

* Sodium p-p'diaminodiphenylsulfone N-N'didextrose sulfonate.

1 Smith, M. I., and McClosky, Wm. T., *Pub. Health Rep.*, 1945, **60**, 1129.

2 Smith, M. I., and Hendrick, E. G., *J. Lab. Clin. Med.*, 1926, **11**, 712.

3 Steinbach, M., *Am. Rev. Tub.*, 1932, **26**, 52.

4 Long, E. R., and Vorwald, A. J., *Nat. Tub. Ass. Tr.*, 1930, **26**, 205.

5 Vorwald, A. J., *Am. Rev. Tub.*, 1933, **27**, 270.

6 Hehre, E., and Freund, J., *Arch. Path.*, 1939, **27**, 289.

7 Oswald, N. C., *Arch. Path.*, 1940, **29**, 678.

8 Wessels, C. C., *Am. Rev. Tub.*, 1941, **43**, 449, 637.

TABLE I.

Group A. Effect of Streptomycin and Promin on Rat Tuberculosis. Sept. 17 5 mg Tubercle Bacilli, Human Strain A27, Injected Intraperitoneally. Treatment: Streptomycin 50,000 Units per kg Intramuscularly Once a Day and Promin 0.7% in the Diet, Both 35 Days.

No.	Days	Tissue smears	Lung subcultures	Subinoculation in guinea pigs PPD	Organs affected	T.B. index
1	28	—	—	2+	Glands, spleen, liver	7
2	30	—	+	—	None	0
3	32	—	+	—	"	0
				—	"	0
4	35	—	—	—	"	0
				—	"	0
5	37	—	—	—	"	0
				—	"	0
6	38	—	—	+	Glands, spleen	3
				—	None	0
7	42	—	—	—	"	0
				—	"	0
8	44	—	+	—	"	0
				—	"	0
				—	"	0
				+	Glands	1
9	46	—	—	+	"	1
				—	None	0
				+	Glands, spleen	4
10	49	—	—	—	None	0
				—	"	0
11	52	—	—	—	"	0
12	53	—	—	—	"	0
				+	None	0

TABLE II.

Group B. Effect of Promin on Rat Tuberculosis. Sept. 17 5 mg Tubercle Bacilli Human Strain A27 Injected Intraperitoneally. Promin Fed 0.7% in the Diet Throughout the Experimental Period.

No.	Days	Tissue smears	Lung subcultures	Subinoculation in guinea pigs PPD	Organs affected	T.B. index
1	28	+	+	+	Omentum, spleen, liver	4
2	30	+		+	Liver	1
3	32	+		+	None	0
				+	"	0
4	35	—	+	+	Spleen	1
				+	Omentum, spleen, liver	4
5	37	+		3+	Glands, spleen, lung	6
				2+	" "	4
6	38	+	+	2+	" " lung	7
				—	" "	3
7	42	+	+	2+	Omentum, spleen, liver	7
				+	Glands, lung	2
8	44	—		—	None	0
				+	Omentum, spleen, lung	8
				+	" " "	6
9	46	+		+	Glands, spleen	4
				+	" lung	3
				+	" " omentum	3
10	49	—	+	2+	" liver	3
				+	" spleen	3
				—	" "	2
11	52	+	+	—	"	1
				+	" spleen	3
				+	"	2
12	53	+	+	2+	" liver	3
				—	" spleen	3
				—	"	1

TABLE III.

Group C. Effect of Streptomycin on Rat Tuberculosis. Sept. 17 5 mg Tubercle Bacilli Human Strain A27 Intraperitoneally. Streptomycin Treatment 50,000 Units per kg Intramuscularly Once a Day for 35 Days.

No.	Days	Tissue smears	Lung subcultures	Subinoculation in guinea pigs		
				PPD	Organs affected	T.B. index
1	28	—	—	—	None	0
2	30	—	+			
3	32	—	—	2+	Glands, spleen, lung	5
4	35	—	+	2+	Liver	1
				2+	Omentum, lung	3
5	37	—	—	2+	Glands, spleen, liver, lung	7
				±	Omentum, spleen, lung	3
6	38	—	+	+	Glands, spleen	5
				+	Omentum, glands, spleen, lung	7
7	42	—	+	—	None	0
				—	,,	0
8	44	+	+	+	Glands, liver, spleen	5
				+	,, ,, ,,	5
				+	Omentum, liver, spleen, lung	9
				+	Glands, liver, spleen	5
9	46	—	+	3+	,, ,, ,, lung	4
				+	,, ,, ,, ,,	8
				+	,, ,, ,, ,,	6
				—	,, ,, ,, ,,	4
10	49	—	—	+	None	0
				—	,,	0
				+	Glands	2
11	52	+	+	2+	,, liver lung	5
				+	,, ,, ,,	4
				+	,, ,,	6
12	53	—	+	+	,, liver, spleen	3
				±	,, omentum	2
				±	,, spleen, lung	4

strain A27. Immediately following inoculation treatment was begun and continued as follows:

Group A. Streptomycin 50,000 units per kg injected intramuscularly once daily and promin fed in the diet at a level of 0.7%. This treatment was continued 35 days.

Group B. Promin fed in the diet at a level of 0.7% to the end of the experiment when the animals were killed.

Group C. Streptomycin 50,000 units per kg as in group A, but no promin. This was continued as in group A for 35 days.

Group D. Untreated controls.

At 28 to 53 days following inoculation the animals were killed by decapitation in sets of 4, one from each group, and under aseptic conditions the left lobes of the lungs were removed, finely minced with iris knives, macerated with 5 cc sterile saline and centrifuged. After removing the coarse particles the centrifugate was resuspended in the saline and 0.1 cc of this was planted on each of several glycerine-egg slants, while 1.0 cc of the same suspension was injected subcutaneously into the right groin to each of several guinea pigs. Smears were made at the same time from the lungs, liver, spleen and kidney for Ziehl-Neelsen stain and microscopic examination.

The slants were examined for growth at regular intervals, and after an incubation period of from 38 to 63 days the colonies if present were counted and an average for each lung suspension obtained. At from 30 to 45 days following the subinoculation of the lung suspensions into the guinea pigs they were tuberculin tested, using 0.01 mg PPD intracutaneously, and 3 days later they were killed with chloroform, autopsied, and the extent of tuberculous involvement (tuberculosis index) rated on the basis of 0 to 4 in the organs of predelection *viz*, glands, omentum, liver, spleen and lungs, as previously described.[9]

Results. These are given in detail in Tables

[9] Smith, M. I., Emmart, E. W., and Stohlman, E. F., *Am. Rev. Tub.*, 1943, **48**, 32.

TABLE IV.

Group D. Sept. 17 5 mg Tubercle Bacilli Human Strain A27 Intraperitoneally. No Treatment.

No.	Days	Tissue smears	Lung subcultures	Subinoculation in guinea pigs		
				PPD	Organs affected	T.B. index
1	28	+	—	+	Omentum	1
2	30	—	+			
3	32	+	+	+	Glands, liver	2
4	35	+	+		"	2
5	37	+	+	2+	" liver, spleen	7
					" omentum, spleen, lung	7
6	38	+	+	±	" spleen	5
				+	" "	4
7	42	—	+	+	" liver	3
				2+	" spleen	6
8	44	+	+	+	" omentum, liver, lung	6
				2+	Omentum, liver, spleen	7
				+	" " " lung	10
				2+	" " " "	5
9	46	+	+	2+	Glands, spleen	5
				+	" "	3
				—	" " liver	6
				+	" "	3
10	49	—	+	+	" "	3
				±	"	2
11	52	+	+	2+	Omentum, spleen, liver, lung	6
				2+	" liver, lung	5
				3+	" " spleen	9
12	53	—	+	—	Glands	2
				—	"	1

I to IV. The rats in group A treated with streptomycin and promin, shown in Table I, had no acid fast organisms in the tissues as determined by microscopic examination of the smears. The lung suspensions of only 3 of the animals yielded positive cultures in the form of a few colonies, and these did not appear until 45 to 50 days following planting as shown in Table V. Subinoculation of the lung suspensions in guinea pigs indicated that only 5 of the animals of this group harbored pathogenic bacilli, while 5 of the 12 rats (41.6%) appear to have been free from tubercle bacilli by all the tests used. The average tuberculosis index of all the subinoculated guinea pigs in this group was only 0.7.

The animals of group B treated with promin alone to the termination of the experiment harbored viable and pathogenic bacilli in the tissues since all the culture tubes, except those that were contaminated, showed good growth, and all but one of the subinoculated guinea pigs developed macroscopic tuberculosis with an average tuberculosis index of 3.1. The results in this promin-treated group were not much better than in the control group D, detailed in Table IV, in which all but one of the cultures were positive and all the subinoculated guinea pigs were positive with an average tuberculosis index of 4.6.

Blood level determinations for promin in the animals of groups A and B done at necropsy revealed a mean concentration of 4.4 mg % with a variation of 2.9 to 7.6 mg % in the animals of the A group, and a mean concentration of 4.1 mg % with a variation of 1.8 to 13.0 mg % in the animals of the B group.

The results of treatment with streptomycin alone in group C as shown in Table III, were somewhat better than in the controls or in the animals treated with promin, but not nearly as good as in the animals receiving the combined treatment. The subcultures of the lung suspensions in group C were positive in 8 of the 12 animals, and the subinoculation tests in guinea pigs indicated 9 of the 11 animals to harbor pathogenic organisms. The guinea pigs subinoculated with the lung suspension of one of the rats of this group (No. 2) died too soon after inoculation. The

TABLE V.
Data of Subculture Tests of Lung Suspensions on Glycerine-Egg Slants. Maximum Incubation period 63 days.

Rat No.	Interval from inoculation of slants to appearance of colonies, days				Avg No. of colonies at end of incubation period			
	Group A promin and streptomycin	Group B promin	Group C streptomycin	Group D control	Group A promin and streptomycin	Group B promin	Group C streptomycin	Group D control
1	*	49	*	*	0	4	0	0
2	47	†	47	47	0.5	†	6	3
3	45	†	*	38	0.5	†	0	10
4	*	12	35	35	0	12	0.2	40
5	*	†	*	10	0	†	0	200
6	*	19	32	19	0	200	12	33
7	*	15	35	28	0	187	0.5	200
8	50	†	26	26	0.3	†	150	175
9	*	†	24	24	0	†	150	150
10	*	28	*	21	0	7	0	7
11	*	25	25	25	0	12	17	17
12	*	24	24	17	0	75	13	4

* No visible colonies after 63 days incubation.
† Contaminated.

TABLE VI.
Summary of All Tests.

Group	Positive smears	Positive subcultures	Guinea pig subinoculation tests: Positive PPD	Macroscopic tuberculosis	Avg T.B. index
Streptomycin and promin	0/12	3/12	5/12	5/12	0.7
Promin	9/12	7/7*	12/12	11/12	3.1
Streptomycin	2/12	8/12	9/11	9/11	3.8
Controls	8/12	11/12	10/11	11/11	4.6

* Five contaminated.

average tuberculosis index for the whole group of subinoculated guinea pigs in this series was 3.8.

Table V gives the detailed data on the subculture tests of the lung suspensions on glycerine-egg slants. Comparison of the average number of colonies on the slants at the end of the incubation period in the several groups indicates marked reduction on the slants planted with lung suspensions from the combined treatment group A, slight reduction in the case of the streptomycin group C, and little difference as between the promin-treated group B and the controls of group D. The relatively low counts on the slants from the first 3 animals of the series, sacrificed at from 28 to 32 days after incubation, may be accounted for by the relatively shorter infection period, while the low counts in the last 3 animals of the series is probably due to the relatively shorter incubation period between the inoculation of the slants and the counting of the colonies.

A summary tabulation of the results of all the tests employed in each of the four groups is given in Table VI.

Discussion. The data shown in Table VI, summarizing the results of all the tests, indicate that the combined treatment with streptomycin and promin was moderately effective in eradicating the tubercle bacilli from the tissues or in preventing their multiplication. Neither streptomycin nor promin alone had any such effects. The former may have exerted a slight bacteriostatic action as evidenced by the reduced colony counts when compared with the controls. Promin alone had no deterrent effect. These findings harmonize well with the previously observed synergistic action of streptomycin and promin in the chemotherapeutic treatment of experimental tuberculosis in guinea pigs and the

relative ineffectiveness of promin alone.[1] It was previously suggested that the slight retardation of the tuberculous process in guinea pigs by promin is probably due to an attenuating action on the tubercle bacillus.[10] The present experiments appear to indicate a slight bacteriostatic action by streptomycin against the tubercle bacillus *in vivo,* and a tuberculocidal action by the simultaneous application of streptomycin and promin. The mechanism of the combined action of the 2 drugs still remains to be determined.

It may be of interest to point out the relative value of the 3 tests employed to determine the presence of tubercle bacilli in the tissues. By direct smear examinations 19 or 39.7% of the 48 infected rats in the 4 groups gave positive results, by the subculture technic 29 or 60.4% were positive, and by the guinea pig subinoculation tests 36 or 75% were positive.

Summary and Conclusions. Rats inoculated with a human strain of tubercle bacilli were treated with streptomycin and promin, individually and in combination, and the effect of treatment determined by (a) direct tissue smears, (b) subculturing of lung suspensions, (c) subinoculation of lung suspensions in guinea pigs. Treatment with promin alone showed no beneficial effects, treatment with streptomycin alone resulted in an average lower colony count than in the controls, while treatment with both drugs appeared to indicate sterilization of 41.6% of the animals and marked decrease and attenuation of persisting viable tubercle bacilli in the remainder.

10 Emmart, E. W., and Smith, M. I., Proc. Soc. Exp. Biol. and Med., 1942, **50**, 320.

15407

Streptomycin and Penicillin Resistant Staphylococci; Influence of pH, Body Fluids on Streptomycin Action.

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Interest in streptomycin has grown rapidly since its discovery by Schatz, Bugie, and Waksman.[1] The purpose of this report is to present experimental observations upon the development of a streptomycin- and penicillin-resistant strain of *Staphylococcus aureus.*

Materials and Methods. The *Staphylococcus aureus* used was the Smith strain.* Its viability was maintained by daily transfer in F.D.A.† broth, and it was found to be sensitive to 1.0 to 2.0 units of streptomycin per cc of medium. The same broth of pH 7.5 was employed for all sensitivity and culture studies. Streptomycin hydrochloride‡ of one lot number was used throughout the study. Streptomycin sensitivity tests were done by the serial broth dilution method, and assays by the agar cup plate method of Stebbins and Robinson.[2]

Organisms Naturally Resistant to Streptomycin. In making routine streptomycin assays by the cup plate method, it was noted that a few isolated colonies of the test organism, the Smith *Staphylococcus aureus,* grew within the clear zone of inhibition produced by the drug. These colonies, when picked and subcultured, produced organisms that were about 10 times more resistant to

1 Schatz, A., Bugie, E., and Waksman, S. A., Proc. Soc. Exp. Biol. and Med., 1944, **55**, 66.

* Received from Merck and Co., Rahway, N. J.

† Formula:

Peptone (Siccum, Armour)	10.0
Bacto Beef Extract (Difco)	5.0
NaCl	2.5
Distilled H_2O	ad 1000 cc

‡ Streptomycin hydrochloride, lot No. 165, kindly supplied by Merck and Co., through the courtesy of Drs. Carlisle and Robertson.

2 Stebbins, R. B., and Robinson, H. J., Proc. Soc. Exp. Biol. and Med., 1945, **59**, 255.

TABLE I.
Effect of Treatment with Streptomycin and Penicillin on Mice Infected with Sensitive and Resistant Strains of *Staphylococcus aureus* (Smith).

Infecting organism	Treatment	No. of mice	No. of mice surviving			
			24	36	48	72 hr
Staphylococcus aureus (Smith) Stock culture	None	5	2	1	0	0
	Penicillin	5	5	5	5	5
	Streptomycin	5	5	5	5	5
Staphylococcus aureus (Smith) Streptomycin Resistant	None	5	4	2	1	1
	Penicillin	5	5	5	5	5
	Streptomycin	5	5	5	0	0

streptomycin than the original stock culture of the Smith strain microorganisms.

Production of Streptomycin-Resistant Organisms. Streptomycin-resistant organisms could be produced at will by growing the sensitive Smith strain in serial transfers in broth containing increasing amounts of the drug. After 17 such passages, over a period of 30 days, such organisms grew well in broth containing 2500 units of streptomycin per cc.

Penicillin Sensitivity Tests. Titrations in broth were then made to note the sensitivity of the stock Smith organisms to penicillin. They were found to be sensitive to 0.1 to 0.2 units of penicillin per cc. The streptomycin-resistant organisms were equally sensitive to penicillin.

In Vivo Treatment with Penicillin and Streptomycin. A mouse experiment was done to determine the effect of treatment with both streptomycin and penicillin upon infections produced by the streptomycin-sensitive and streptomycin-resistant strains of the Smith *Staphylococcus aureus.* The mice were infected intraperitoneally with 1 cc of a 10-hour broth culture of each of these 2 strains, diluted 5 times in 5% gastric mucin.[3,4] One-third of each group of 15 were then treated with penicillin (250 units I.P. at the time of inoculation, repeated once in 4½ hours), another one-third with streptomycin (500 units I.P. at time of inoculation, repeated once in 4½ hours), and the remainder served as untreated controls.

From Table I it will be observed that an infection produced by a lethal dose of organisms made resistant to streptomycin can be favorably influenced by treatment with penicillin, while relatively large amounts of streptomycin remain quite ineffective.

Miller and Bohnhoff[5] recently published data for the gonococcus and meningococcus demonstrating the same relationships between streptomycin- and penicillin-resistant organisms of these species that we have just described for the staphylococcus.

Production of Penicillin-Resistant Organisms. It proved to be easier to adapt the *Staphylococcus aureus* to increasing concentrations of streptomycin than to penicillin. It took 39 passages over 45 days to make the organisms grow in broth containing 500 units of penicillin per cc and only 15 passages to have a similar effect with streptomycin. No difference was noted in the rate of adaptability to penicillin between the stock culture and the streptomycin-resistant strain.

The organisms made resistant to 2500 units of streptomycin retained their ability to grow well in at least 1000 units per cc of medium after 4 months in the refrigerator, after several rapid mouse passages, and after adapting the organisms to penicillin.

Using the agar plate method of Gots,[6] no substance capable of destroying streptomycin ("streptomycinase") could be demonstrated either in organisms made resistant to 2500 units of the drug, or in the naturally resistant organisms picked from a colony growing with-

[3] Nungester, W. J., Jourdonais, L. F., and Wolf, A. A., *J. Infect. Dis.*, 1936, **59**, 11.

[4] Ercoli, N., Lewis, M. N., and Harker, E., Proc. Soc. Exp. Biol. and Med., 1945, **59**, 273.

[5] Miller, C. P., and Bohnhoff, M., *J. A. M. A.*, 1946, **130**, 485.

[6] Gots, J. S., *Science*, 1945, **102**, 309.

TABLE II.
Concentrations of Streptomycin Necessary for Complete and Partial Inhibition of Growth at Different pH Levels. Incubation 24 hr.

	pH	Concentration of streptomycin in units per cc for	
		Complete inhibition	Partial inhibition
Staphylococcus aureus (Smith)	7.7	1.6	0.8
	7.2	6.25	1.6
	6.6	6.25	3.2
	5.9	50.00	25.0
	5.2	100.00	40.0

Inoculum 0.1 cc 12-hr broth culture diluted 10 times.

in the clear zone of a streptomycin assay plate.

Effect of pH on the Action of Streptomycin. It has been demonstrated that the action of streptomycin varies markedly with change in pH.[7,8] Tubes of F.D.A. broth were adjusted to the following pH's: 5.2, 5.9, 6.6, 7.2 and 7.7, and to these was added enough streptomycin to give concentrations of 0.4 to 200 units per cc. Each tube was then inoculated with 0.1 cc of a 12-hour broth culture of staphylococcus, diluted 10 times.

Table II shows that the inhibitory efficiency of streptomycin in broth diminishes with increasing acidity.

To determine whether the drug is actually destroyed in acid solution, tubes of broth of a pH of 3.5, 6.4 and 7.2 were prepared to contain 10 units of streptomycin per cc. After standing for 2½ hours in the incubator, the tubes were quickly adjusted to pH 7.8 with N/1 NaOH, and streptomycin assays were made. All were found to contain about 10 units per cc, indicating that there had been no destruction of the drug.

Effect of Body Fluids, Pus, and Tissue Juice on Streptomycin. Four groups of broth tubes were set up. To the first group was added 6% by volume of purulent pleural fluid from an uncontaminated tuberculous empyema; to the second, the same volume of a sterile non-purulent tuberculous empyema fluid; to the third, the same volume of an opalescent sterile ascitic fluid; and the fourth group constituted the broth controls. Enough streptomycin solution was then added so that each group contained concentrations of the drug graded from 0.8 to 10 units per cc. The total volume in each tube was 5 cc. All of them were then inoculated with 0.1 cc of a 12-hour broth culture of staphylococcus, diluted 10 times. After 24 and 48 hours incubation at 37°C, each tube was examined for evidence of growth by visual turbidity, and one loopful of broth from each was streaked on an agar plate. The reaction in each group of tubes was checked after incubation, and the pH found to be between 7.2 and 7.5 in all. The results after 48 hours incubation are given in Table III.

It can be seen from the table that 7.5 units of streptomycin per cc killed enough organisms in all 4 groups of tubes to prevent growth on subculture of a loopful of broth, and that 2 to 3 units per cc was sufficient to inhibit growth in the presence of all these exudates. Pus and body fluids, therefore, do not interfere appreciably with the bacteriostatic or bactericidal action of streptomycin in broth.

In another experiment, a streptomycin solution of 100 units per cc was diluted 10 times in tubes of ascitic fluid, purulent pleural fluid, guinea pig serum, tissue juice from livers and spleens of normal guinea pigs, and a saline control. These mixtures were then incubated at 37°C for 24 to 72 hours and then tested for streptomycin activity. When compared with the saline control, there was no significant decrease in activity in any of the above material.

Summary and Conclusions. 1. Certain organisms of the Smith strain *Staphylococcus*

[7] Waksman, S. A., Bugie, E., and Schatz, A., *Proc. Staff Meet. Mayo Clin.*, 1944, **19**, 537.

[8] Loo, Y. H., Skell, P. S., Thornberry, H. H., Ehrlich, J., McGuire, J. M., Savage, G. M., and Sylvester, J. C., *J. Bact.*, 1945, **50**, 701.

TABLE III.
Effect of Body Fluids and Pus on the Action of Streptomycin *in Vitro*. Incubation 48 hr.

Medium	Growth by visual turbidity. Units of streptomycin per cc							Growth by subculture.‡ Units of streptomycin per cc					
	1	2	3	4	5	7.5	10	2	3	4	5	7.5	10
Broth (control)	G*	0	0	0	0	0	0	+†	+	+	—	0	0
Broth + purulent pleural fluid	G	0	0	0	0	0	0	+	—	+	—	0	0
Broth + non-purulent pleural fluid	G	G	0	0	0	0	0	—	+	+	—	0	0
Broth + ascitic fluid	G	G	0	0	0	0	0	+	—	+	—	0	0

* G indicates visual turbidity.
† + indicates growth on subculture; 0 indicates no growth; — indicates tube not subcultured.
‡ One double loopful streaked on surface of agar plate.
Inoculum: 0.1 cc 1:10 dilution of 12-hour broth culture of *Staphylococcus aureus* Smith.

aureus possess a natural increased resistance to streptomycin. This resistance is not based on the production of a "streptomycinase." 2. A strain of staphylococcus, highly resistant to streptomycin, can be produced *in vitro* from the streptomycin-sensitive organisms. 3. Streptomycin resistance is a relatively permanent characteristic, and is not affected by several passages through mice. The resistant organisms retain their virulence for this host. 4. Streptomycin resistance and penicillin resistance are independent of each other. Staphylococci originally penicillin-sensitive, retain their sensitivity to penicillin after being made resistant to streptomycin, and vice versa. Treatment with relatively large amounts of streptomycin is ineffective in mice infected with a streptomycin-resistant strain of *Staphylococcus aureus*. These mice can be cured, however, with penicillin. 5. The bacteriostatic effect of streptomycin diminishes considerably as the acidity of the culture medium increases from pH 7.7 to 5.2; the greatest diminution in effect occurs between pH 6.6 and 5.9. This must be taken into consideration in making *in vitro* assays and sensitivity tests, as well as in evaluating the action of the drug *in vivo*. 6. Streptomycin is not destroyed after contact with broth of pH 3.5 for 2½ hours. 7. Streptomycin is not destroyed, nor are its bacteriostatic and bactericidal powers appreciably influenced, by serous body fluids, pus, or normal tissue juices.

15408

Electrophoretic and Allergenic Analyses of Fractions of Larvae of *Trichinella spiralis*.

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Fractions of larvae of *Trichinella spiralis* have been employed as diagnostic aids in detecting *Trichinella* infection in man. Bachman[1] used an acid-hydrolyzed, saline-soluble extract of *Trichinella* larvae to study intradermal responses in sensitized rabbits. Bozicevich[2] prepared an antigen for precipitin tests from the supernatant fluid of a 5% saline extract heated at 58°C for one hour to precipitate inert material; the same material, diluted and sterilized by intermittent

[1] Bachman, G. W., *J. Prev. Med.*, 1923, **2**, 169.

[2] Bozicevich, J., *Pub. Health Rep.*, 1938, **53**, 2130.

heating, was used without a preservative as an allergenic agent. Melcher[3] separated 6 fractions of larvae and found the acid-soluble fraction to be a potent allergen. This fraction was prepared by alkaline extraction of dehydrated, defatted larvae from which the acid-insoluble non-reactive substances had been removed.

In the present study a comparison was made of electrophoretic and allergenic properties of acid-soluble and of heat-treated fractions with those of the parent saline extract of *Trichinella* larvae. An electrophoretic analysis was made of each fraction in the modified Tiselius apparatus by the moving boundary method.[4] The allergenic properties of the fractions were compared in sensitized rabbits and guinea pigs.

Procedure. Obtaining larvae. About 5,000 larvae suspended in 20% gelatin were fed to each guinea pig by means of a syringe connected to a rubber catheter. Approximately 7 weeks later, the animals were killed and the skinned and eviscerated carcasses were ground twice in a meat chopper. The muscle was digested by the method described by Bozicevich[2] and modified by Line.[5] Approximately 150 g of ground material were placed in a 2 liter beaker and covered with warm digestion fluid which contained 2.5 g of pepsin (activity 1:3,000) and 7 ml hydrochloric acid (sp. gr. 1.18) in one liter of water. Twelve thicknesses of 44 mesh gauze were tied around the top of the beaker. A funnel of 2 liter capacity was connected to a centrifuge tube with rubber tubing. The funnel was almost filled with the digestion fluid. The beaker containing the muscle was covered with a piece of paper and quickly inverted into the funnel. At this point, the beaker rested in an inverted position with the level of the digestion fluid in the funnel above the rim of the beaker. The paper was withdrawn carefully to avoid unnecessary stirring. As digestion proceeded at 37°C for 5-7 hours, the larvae settled to the bottom of the centrifuge tube. The freed larvae were alternately washed with distilled water and centrifuged 6 times, frozen at —12°C, dried under vacuum from the frozen state and sealed under vacuum.

Extraction. A weighed amount of dried larvae was ground in sterile 0.85% salt solution in a sterile tissue grinder and diluted to a 5% suspension. The material was held overnight at 4°C, adjusted to pH 7.0 and centrifuged at 3,500 r.p.m. (2,200 × gravity calculated from the radius at the base of the angle head). The supernatant fluid is designated as the primary extract.

Acid treatment. One portion of the extract was adjusted to pH 4.8 with 0.2 N hydrochloric acid in a Beckman pH meter and held at 4°C overnight. Insoluble material was removed by centrifugation and the supernatant fluid designated as the acid-soluble fraction. The sample for intradermal injections was adjusted to neutrality with 0.1 N sodium hydroxide. This fraction was not exactly comparable to the acid-soluble fraction analyzed by Melcher.[3]

Heat treatment. A second portion of the extract was heated at 56°C for an hour. This was likewise centrifuged and the supernatant fluid referred to as the heat-treated fraction.

Electrophoretic technic. For electrophoretic analysis the samples were placed in viscose tubing and dialyzed at 3°C against large volumes of phosphate buffer of pH 6.8 and ionic strength 0.03. Electrophoresis was carried out at 0°C and the schlieren scanning method of Longsworth[6] was used in photographing the boundaries.

Asymmetric boundaries were obtained in all patterns. Since the ascending boundaries swept through larger volumes and a better separation of components was obtained than in the corresponding descending boundaries, all calculations were made from ascending patterns. In computing relative areas it was assumed that the immobile peaks in all patterns were δ and ϵ boundaries.

Intradermal tests. Infected rabbits and guinea pigs were used for comparing allergenic potencies. The rabbits had been fed about

[3] Melcher, L. R., *J. Inf. Dis.*, 1943, **73**, 31.

[4] Longsworth, L. G., and MacInnes, D. A., *Chem. Rev.*, 1939, **24**, 271.

[5] Line, C. B., unpublished procedure.

[6] Longsworth, L. G., *Ann. New York Acad. Sc.*, 1939, **39**, 187.

Fig. 1.
Primary extract of larvae of *Trichinella spiralis*. Sample diluted 1-2.7. Electrophoresis for 3225 seconds at 8.87 volts per cm.

Fig. 2.
Acid-soluble fraction. Electrophoresis for 5600 seconds at 7.73 volts per cm.

Fig. 3.
Heat-treated fraction. Electrophoresis for 5700 seconds at 8.81 volts per cm.

20,000 larvae each, 4 to 16 months prior to test, the guinea pigs about 5,000 larvae each, 18 months previously. For testing, samples of the 3 fractions were filtered through a sterile, fine porosity fritted-glass filter and diluted. A sterility test on each sample was satisfactory. Dilutions of 1:10,000 based on the original dry weight of whole larvae were made for injections into rabbits and 1:1,000 for guinea pigs. Animals were injected intradermally with 0.05 ml and observed hourly. The maximum reaction usually occurred in 2 to 5 hours. The reactions were recorded in terms of the diameters of the areas of edema.

Results and Discussion. Electrophoretic analysis. Extraction and fractionation were carried out on 2 lots of *Trichinella* larvae. The electrophoretic patterns of Lot 1, shown in Fig. 1, 2 and 3, are also typical of the patterns of Lot 2. The difference in the total concentration of the primary extracts may be attributed to possible variations in the extraction and in centrifugation.

In the patterns of the primary extract of *Trichinella* larvae (Fig. 1) 3 homogeneous peaks are discernible. The area between peaks 1 and 2 is not a separate component and cannot be defined. More than 60% of the total primary extract is represented by the heterogeneous mixture designated as IV. The mobility calculated for this mass is an average value (Table I).

The patterns of the acid-soluble fraction are shown in Fig. 2. Three electrophoretically separable components were present, in addition to the heterogeneous mixture which had the highest mobility and an incompletely separated portion migrating between components 2 and 3. Physico-chemical changes produced by the acid treatment may be noted by comparing Fig. 1 and 2. The mobility and concentration of component 1 in the acid-soluble fraction are not significantly different from those of the corresponding component 1 in the primary extract. There were modifications in component 2 and the portion closely associated with it as shown by changes in concentration and in the mobility of the homogeneous peak. The most significant effect of the acid treatment is noted in the fast moving heterogeneous mass designated as IV. Computations of concentrations using

TABLE I.
Electrophoretic Analysis of Extracts of *Trichinella* Larvæ.

Fraction	Mobility, $\mu \times 10^5$ cm^2				Relative composition, % total areas				Concentration, g/100 ml				
	1	2	3	IV	1	2	3	IV	Total	1	2	3	IV
Primary extract													
Lot 1	2.1	4.2	5.8	7.8	6	13	15	62	4.00	0.24	0.52	0.61	2.48
" 2	1.8	4.7	5.9	8.0	12	5	13	65	2.76	0.32	0.14	0.36	1.80
Acid-soluble													
Lot 1	1.7	3.4	5.9	7.2	27	16	24	9	1.23	0.33	0.20	0.29	0.11
" 2	1.4	2.7	5.4	7.5	27	16	36	6	1.58	0.43	0.25	0.57	0.10
Heat-treated													
Lot 1	1.4	3.3	5.5	6.6	33	12	27	8	0.67	0.22	0.08	0.18	0.05

TABLE II.
Intradermal Reactions of Fractions of Larvæ of *Trichinella spiralis*.

Fraction	Size of reaction (mm)			Ratios of areas		Ratio of concentrations
	Smallest	Largest	Mean	Mean	Standard error	
	7 series of tests in rabbits.					
Primary extract	10 x 10	15 x 20	13.0 x 14.9	—	—	—
Acid-soluble	11 x 12	16 x 18	13.0 x 15.0	1.05	0.10	0.31
Heat-treated	7 x 10	14 x 15	11.0 x 12.6	0.74	0.08	0.17
	25 series of tests in guinea pigs.					
Primary extract	8 x 9	16 x 18	12.0 x 13.4	—	—	—
Acid-soluble	8 x 10	18 x 18	12.0 x 13.0	0.99	0.04	
Heat-treated	7 x 8	16 x 16	11.0 x 11.8	0.83	0.04	

TABLE III.
Relation of Concentrations of Components to Skin Reactions.

Fractions of Lot 1	Ratios of areas		Ratio of the concentrations of components		
	Rabbits	Guinea pigs	Component 1	Component 2	Component 3
Acid-soluble	1.05	0.99	1.4	0.4	0.5
Heat-treated	0.74	0.83	0.9	0.15	0.3

relative areas and dry weight determinations show that the material removed by acid treatment may be accounted for primarily by the decrease in portion IV.

The boundaries in the electrophoretic patterns of the heat-treated sample in Fig. 3 are very similar to those in patterns of the acid-soluble fraction. Three components separated and the 2 heterogeneous portions are present in the same positions as in Fig. 2. About 80% of the primary extract was removed by heating. Component 1 was apparently stable at the temperature and time interval used; there was some decrease in components 2 and 3 but the marked decrease in the heterogeneous mass IV was equivalent to approximately 75% of the total loss.

Intradermal reactions. Seven rabbits and 16 guinea pigs were injected with each of the 3 allergens. Prior to feeding of larvae, the rabbits did not respond to injections of primary extract. To confirm the specificity of the response, 14 normal guinea pigs were injected with each of the 3 allergens; there were no reactions. The saline control induced transitory responses in 2 of the 16 infected guinea pigs. The reactions were 3 × 3 mm, appeared later and persisted a shorter time than those to the fractions of larvae. Twenty-five series of tests were made on the infected guinea pigs with alternation in the location of injections.

The individual variations among the infected animals in response to an allergen were approximately the same when any one of the allergens was tested. In Table II are recorded the areas of smallest, the largest and the average reaction in the animals. The ratio of the edematous areas produced by the allergens and the ratio of dry weights were calculated by using the primary saline extract as the reference standard.

The acid treatment of the primary extract of the larvae produced no detectable alterations in properties which elicit dermal responses although 70% of the material had been removed. The electrophoretic patterns showed a significant decrease only in the heterogeneous mass designated as IV.

There was an indication of some decrease in the area of intradermal reactions produced from the injection of the antigen prepared by heating the primary extract. Most of fraction IV, as well as some of components 2 and 3, was removed by heating.

Computed on the basis of dry weight of soluble material present, the dilutions of the samples tested in sensitized rabbits were: Primary extract, 1:20,000, acid-soluble, 1:60,000; and heat-treated, 1:100,000. The decrease in the area of allergic reaction to the heat-treated fraction was relatively small in comparison to the dilution factors.

In Table III the concentration of each com-

ponent is compared with the skin activity of each fraction. The ratio of the concentration in grams per 100 ml (Table I) of each component was calculated by using the amount of each component in the primary extract as the reference standard for that component. If the ratios of concentration of the components are compared with the ratios of the areas of reactivity in skin tests (Table II) it will be noted that the concentration of component 1 parallels results of skin tests on the fractions. The ratios of the concentrations of components 2 and 3 and of skin activity are not proportional. These calculations indicate a stability of component 1 and suggest that the allergen is associated with this component.

Summary. A saline-soluble portion of larvae of *Trichinella spiralis* has been compared as to its electrophoretic characteristics and production of allergic response in sensitized animals with other fractions, prepared by acidification and by heating. Each of the 3 fractions separated into 3 electrophoretic components in addition to an incompletely separated portion associated with component 2 and a fast moving heterogeneous mixture. In the primary extract the heterogeneous mass represented more than 60% of the total concentration. Either acid or heat treatment removed practically all of this mixture. More material was precipitated by heating than by acidification. The 3 fractions were diluted on the basis of the original dry weight of larvae and intradermal reactions compared. There was no apparent difference in the area of edema produced by the primary extract and the acid-soluble fraction. The heat-treated allergen caused slightly less reaction than the other 2. There is an indication that the allergen is associated with component 1.

15409

Purified Rations and the Importance of Folic Acid in Mink Nutrition.*

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Domestication of mink has introduced many practical nutritional problems. The use of purified rations which has elucidated practical feeding problems in many other species of animals appears to offer a useful tool in the study of these problems in mink. Establishment of nutritional requirements of an animal so different from those previously studied would also be of considerable academic interest. In this paper we wish to present data on the use of purified rations and the requirement of folic acid for the mink.

Experimental. Previous to being placed on experiment the mink were housed in individual outdoor wire pens. They were fed a ration containing 72% horsemeat (including bone), 10% fresh liver, 12% cereal mixture, and 6% tomato puree. When the animals were placed on experiment the cages were moved into an unheated room and the nest boxes removed. The composition of the purified rations employed is given in Table I. The basal ration was similar to that used for dogs by Schaefer *et al.*[1] All rations were weighed into pound butter crocks, fitted with inverted conical shields in order to reduce spillage. Weekly weight and daily food consumption data were recorded. Blood samples were taken by severing the metatarsal vein according to the technic described by Kennedy.[2] White blood cell counts were determined by the

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

1 Schaefer, A. E., McKibbin, J. M., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 365.

2 Kennedy, A. H., *Ontario Gov't. Exp. Fur Farm, Bul. No. 6*, 1933.

TABLE I.
Synthetic Rations.

	Basal, %	High protein, %	Basal + gelatin, %
Sucrose	66	55	56
Casein—alcohol extracted	19	30	19
Gelatin	—	—	10
Cottonseed oil	8	8	8
Cod liver oil	3	3	3
Salts No. IV	4	4	4

Vitamin Supplements

	Mg per 100 g of ration
Thiamine	0.2
Riboflavin	0.4
Pyridoxine	0.2
Calcium pantothenate	1.5
Niacin	4.0
Choline	100

Vitamins Per Animal Per Day, Added Where Indicated
(Mixed in ration daily)

Folic acid	50 μg
Biotin	50 "
Inositol	20 mg
Para-aminobenzoic acid	50 "
Alpha-tocopherol	2 "
2-methyl-1,4-naphthoquinone	.5 "

usual methods and hemoglobin by the method of Evelyn.[3]

Results. Approximately one-half of the mink began eating the purified ration immediately whereas the remainder were given in addition to the purified ration 20 g of stock meat ration for the first 7 to 10 days. In general 2 weeks were necessary for the mink to become accustomed to the change in ration and the frequent handling.

Growth curves of Mink 9 and 10 placed on the basal ration are shown in Fig. 1. The animals lost weight at first and then showed a rather constant weight. After 10 to 13 weeks weight loss was again severe. This weight loss was accompanied by extreme weakness, bloody, watery feces and irritability. In the case of Mink 9 there was marked anorexia. At this time 250 μg folic acid was fed orally and 50 μg added to the ration daily thereafter. There was an immediate marked improvement in the condition of the animal. In the following 24 hours the entire allotment of the ration was consumed. The diarrhea, weakness and general irritability completely disappeared in a few days. After 14 weeks on this ration Mink 10 died before supplementation was started. Normal food consumption had been maintained until the time of death.

The basal ration was modified by increasing the casein level to 30% at the expense of sucrose (Table I) and 4 mink were placed on this regimen. Growth curves are shown in Fig. 2. At about the ninth week the 4 mink began to lose weight markedly and Mink 16 was fed 100 μg of biotin orally and 50 μg was added to the ration daily. The loss in weight continued and after the biotin supplement had been continued for 2 weeks the previously described syndrome was very evident. At this time folic acid therapy (1.0 mg orally and 50 μg added to the ration daily) was started. An immediate response to this therapy was noted. At the beginning of the 12th week Mink 18 was given biotin at the levels described for Mink 16. Loss of body weight continued. After one week of such therapy the animal appeared normal at 8 A.M. but large quantities of bloody, watery fecal discharge were noted. An hour later the animal was found in a flaccid, morbid condition. Blood samples were taken and the animal autopsied. Mink 15 and 17 exhibited the severe deficiency symptoms in 10 to 11 weeks. Folic acid therapy as described for Mink 16 was started at this time. Symptoms such as weakness, irritability, bloody watery fecal discharge and anorexia disappeared rapidly, with resultant increase in food consumption and body weight. Hemoglobin and white cell count determinations prior to and at the time of critical deficiency and after therapy indicates that the decrease in the white cell count and slight drop in hemoglobin was corrected by folic acid (Table II).

The basal ration was modified by adding 10% gelatin at the expense of sucrose (Table I), and 4 animals placed on this regimen. Weight curves are shown in Fig. 2. All animals began losing weight after 4 to 8 weeks. After receiving this ration for periods of 10 to 13 weeks Mink 1, 3 and 6 were fed orally an initial dose of biotin at levels at 100 μg, 750 μg and 250 μg respectively in addition to starting the daily supplement of 50 μg

[3] Evelyn, K. A., *J. Biol. Chem.*, 1936, **115**, 63.

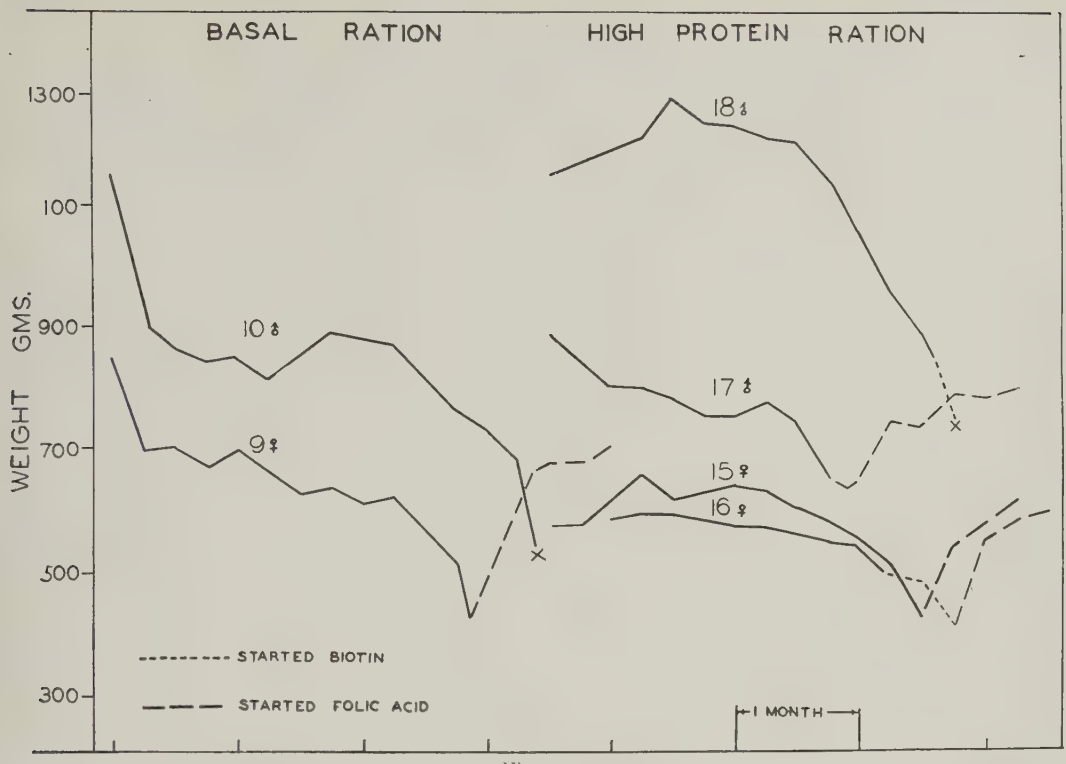

Fig. 1.

Body weight curves of Minks 9 and 10 receiving the basal ration and Minks 15, 16, 17, 18 receiving the high protein ration.

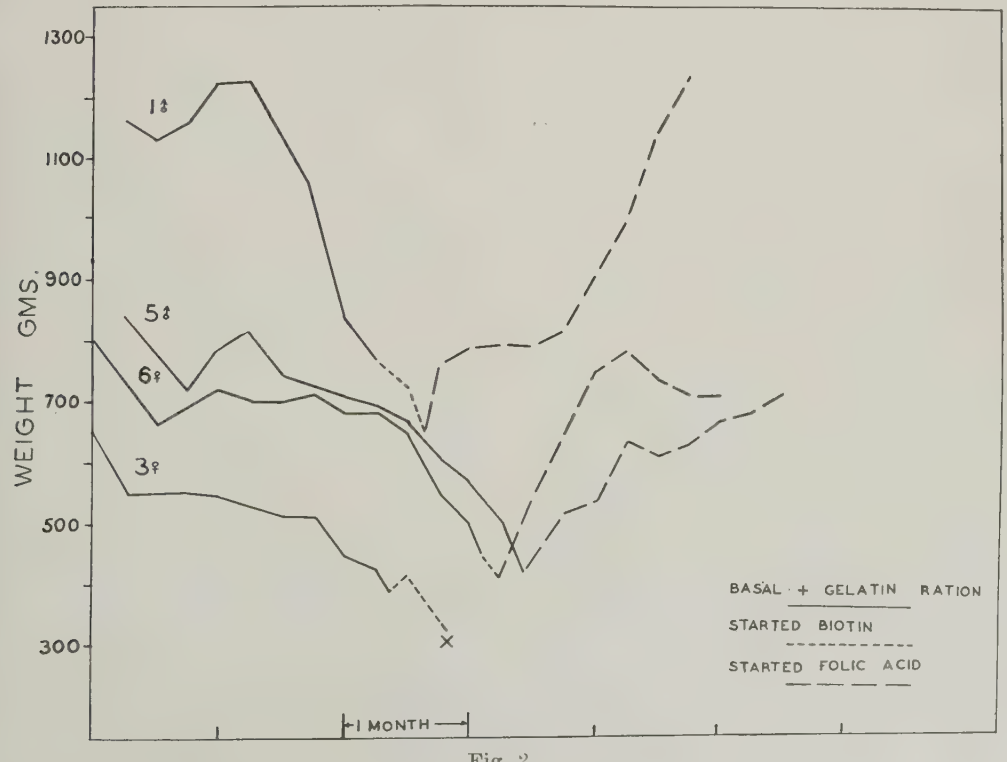

Fig. 2.

Body weight curves of Minks 1, 3, 5, and 6 receiving the basal plus gelatin ration.

TABLE II.
Blood Analysis.

Mink No.	Ration	Total days on exp.	Body wt in g	Hb g%	WBC per comm.	Remarks
4	Basal + gel + 10% fresh liver	120	750	18.7	17,400	
7	Basal + gel + 2% liver fraction "L"	130	500	16.3	14,400	
5	Basal + gel	30	750	19.3	—	
	"	84	420	14.7	7,300	Started folic acid
	"	133	680	19.2	16,000	
6	"	84	410	15.3	—	" " "
	"	135	715	18.0	15,000	
19	"	79	605	16.8	8,800	
		88	505	15.9	6,600	" " "
		97	660	14.3	15,100	
15	High protein	63	540	15.0	9,000	
		77	430	14.3	8,700	" " "
		91	615	18.3	15,300	
16	"	63	500	17.2	8,800	
		77	420	15.7	8,760	" " "
		91	585	18.9	13,400	
17	"	70	655	15.8	8,100	" " "
		103	800	18.7	21,000	
18	"	76	900	17.4	10,000	
		87	700	13.5	6,160	Fatal deficiency

biotin. Loss of body weight continued and severe deficiency symptoms characterized by approximately 50% loss in body weight accompanied by bloody fecal discharge; irritability, general weakness and anorexia developed. At this time Mink 3 died and Mink 1 and 6 were fed 250 μg folic acid orally in addition to starting the daily supplement of 50 μg per day. Biotin was not fed to Mink 5, and after being on this ration for 10 weeks the typical syndrome was noted. 550 μg folic acid was administered orally and the daily supplement started. Immediate remission of deficiency symptoms occurred in the 3 animals receiving folic acid. Blood analysis (Table II) of Mink 5 and 6 indicates that the number of white blood cells was markedly increased and hemoglobin slightly increased after folic acid therapy.

Later in the experiment Mink 19 was started on this same ration. Failure to maintain body weight was noted at the end of the 11th week. At this time para-aminobenzoic acid, inositol, α-tocopherol and 2-methyl-1,4-naphthoquinone were added daily to the ration. Loss of body weight continued and 10 days later the animal exhibited the typical deficiency symptoms noted in the previously described mink. At this time folic acid therapy of 1 mg orally plus the daily supplement was started. Dramatic recovery was noted. The 35% loss of total body weight was regained in a period of 18 days.

Autopsies were performed on Mink 3, 10 and 18. Ulcerative hemorrhagic gastritis with large volumes of blood in the stomach and intestines were noted in all cases. Macroscopically the liver appeared congested; kidney, spleen, and other organs appeared normal.

In conjunction with studies on the purified rations supplemented only with the 6 synthetic vitamins, 5 mink were fed additional supplements of whole liver and liver extracts. Weight curves are shown in Fig. 3. Mink 2, 4, 7 and 8 were started on the basal plus gelatin ration supplemented with various liver fractions. Mink 2 received 2% 1:20 liver

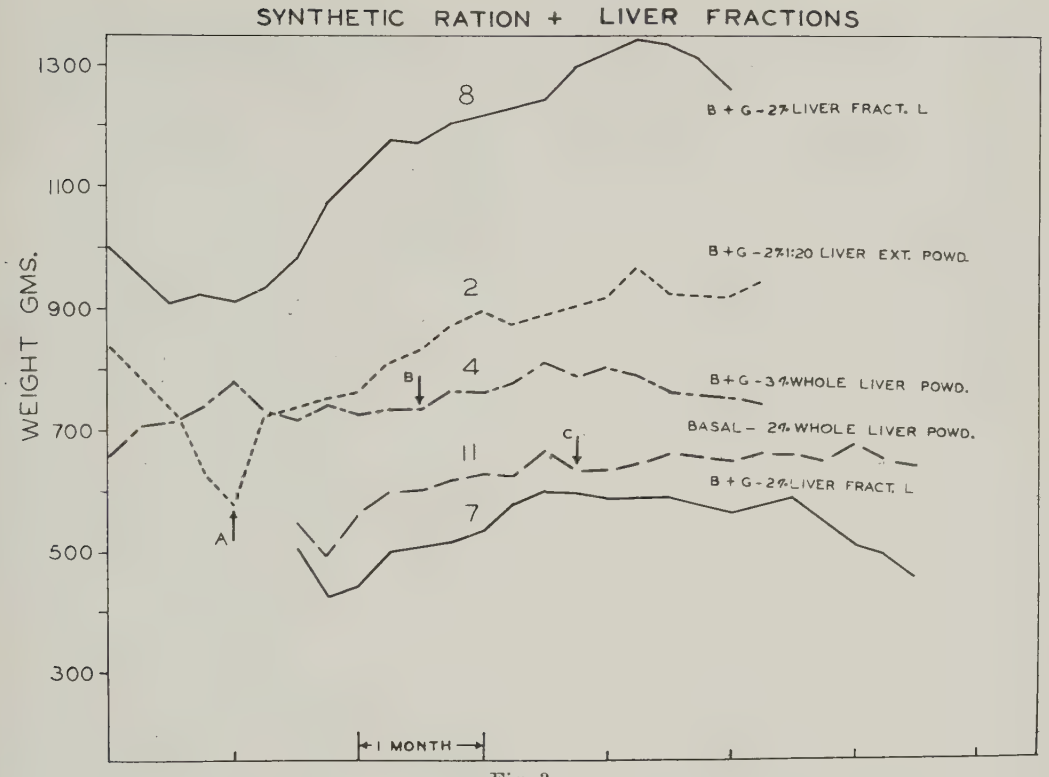

Fig. 3.

Body weight curves of 5 mink receiving purified rations supplemented with various liver concentrates.

B + G—Basal + gelatin ration.

A—Started 50 μg of biotin per day.

B—Changed supplement from 3% whole liver powder to 10% fresh calves liver.

C—Increased whole liver powder from 2% to 4%.

extract powder and lost 30% body weight in 27 days. At this time 50 μg of biotin daily was added to the ration. An immediate response in body weight was noted during the first week. However, remission was slow after this initial gain. In an effort to determine whether fresh liver would increase body weight above that obtained with liver extracts the supplement of 3% dried whole liver substance for Mink 4 was changed to 10% fresh calves liver. The plateau in body weight continued. Although liver concentrates as fraction "L" and dried whole liver powder maintained body weight and produced some additional growth during 5 months a general decline in weight in Mink 7 is now being observed.

Discussion. The use of purified rations for mink introduces another species of animal for experimental nutritional studies.

Eleven mink placed on purified rations containing varying levels of protein supplemented with thiamine, riboflavin, pyridoxine, pantothenic acid, niacin and choline developed critical deficiency symptoms in 9 to 13 weeks. White blood cell counts were reduced to 6,160-8,760. The hemoglobin level was slightly lowered. The administration of folic acid to 8 of the 11 mink resulted in a dramatic recovery from the syndrome characterized by irritability, weakness, bloody fecal discharge, anorexia and severe weight loss. White blood cell counts rose to 13,400-21,000; also a slight increase in hemoglobin was noted. This increase in white blood cells upon administration of folic acid is similar to the response

noted in monkeys as reported by Cooperman *et al.*[4]

Preliminary observation in Mink 1 at the present time indicates that an additional factor in fresh liver is required. After the response in body weight to folic acid therapy this animal lost 40% of the total body weight in a period of 3 weeks. Inositol, *p*-aminobenzoic acid, vitamin E and K at levels given in Table I were added to the ration with no response in body weight. The feeding of 10% fresh liver for 18 days resulted in a regain of 63% of the body weight loss; hemoglobin rose from 10.7 at the time of deficiency to 17.3 g per 100 cc of blood.

The possible role of biotin in the nutrition of the mink is still obscure. A response to biotin in Mink 2 was obtained. However, further efforts to produce an uncomplicated biotin deficiency has been unsuccessful to date.

The role of *p*-aminobenzoic acid, inositol, α-tocopherol and 2-methyl-1,4-naphthoquinone has not been established in our preliminary work.

The practical aspect of these studies indicates the importance of considering the folic acid content of mink rations. Further studies as to the requirement of the known members of the vitamin B complex and additional factors in relation to growth, maintenance, reproduction, and development of fur are necessary.

Summary. 1. Purified rations have been successfully employed in studying nutritional requirements of adult mink. 2. Feeding highly purified vitamin B complex free rations of varying protein levels supplemented with thiamine, riboflavin, niacin, pyridoxine, pantothenic acid and choline produced a severe deficiency in 10 to 13 weeks. The characteristic symptoms are given. 3. The administration of synthetic folic acid to 8 mink at the time of severe deficiency symptoms resulted in an immediate recovery, whereas 3 mink not receiving folic acid died. 4. Preliminary observation indicates the existence of another factor present in liver distinct from the known vitamins which is seemingly necessary for maintenance of body weight and hemoglobin regeneration.

We wish to acknowledge our indebtedness to Merck and Co., Rahway, N. J., for the crystalline vitamins; to Wilson Laboratories, Chicago, Ill., for the various liver preparations; to Dr. B. L. Hutchings of Lederle Laboratories, Inc., Pearl River, N. Y., for the synthetic folic acid.

4 Cooperman, J. M., Elvehjem, C. A., McCall, K. B., and Ruegamer, W. R., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 92.

15410

Toxicity of Choline in the Diet of Growing Chickens.

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Choline has a wide potential use in rations for poultry. Because of the probability of this substance's being fed in amounts greater than needed for maximum benefits, it is desirable that information on the toxicity of the compound for poultry be obtained.

One of the authors (P.B.P.) found that an oral dose of 5 g was fatal to 5 kg rabbits within 30 minutes, whereas with rats, Hodge and Goldstein[1] found that 6.7 g per kg body weight given by stomach tube to rats starved for several hours "represented the LD_{50} for albino rats."

Hodge[2] later reports the results of supply-

1 Hodge, H. C., and Goldstein, Max R., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 281.

2 Hodge, H. C., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 212.

TABLE I.
Effect of Dietary Level of Choline on Growth of Chickens.

	Series 1				Series 2	
Lot No.	1	2	3	4	5	6
% choline added	0.05	1	2	4	0.05	1
Avg gain in g*	1201	1043	1035	915	1089	972
Index of gains, controls 100	100.0	86.8	86.2	76.2	100.0	89.2

* Avg of mean gains of males and females.

ing varying percentages of choline chloride in the feed and in the drinking water of rats over a 4 months' period. Amounts up to 1% in the feed did not retard growth, but the inclusion of 1% or more in the drinking water resulted in reduced growth rates. In his study Hodge was unable to attribute any consistently abnormal histopathological findings to the effect of the choline chloride, and indications were that where the larger percentages of choline were used the resultant retarded growth rates were due to limited intakes of feed and water in the respective series of the experiment.

In the past the possible toxicity of choline to chickens has been given but little consideration. However, the authors, in blood choline studies, had previously fed mature hens about 3% of choline chloride over a 3 months' period and had observed a decline in egg production and a partial molt, the new feathers being curled, and presenting an overall frizzled appearance.

In the present studies the effect of different levels of choline chloride in the diet on the growth and fat deposition in chickens was determined. In the report of this work the terms choline and choline chloride are used interchangeably.

Experimental. One-week-old hybrid chicks (New Hampshire Red × White Leghorn) were divided into 8 even-weight lots of 16 birds each. They were reared according to standard procedure, and the experiment covered a period of 11 weeks. The experiment was conducted with 2 series of diets. The basal rations contained 0.05% of choline chloride which is considered adequate to meet the needs for maximum growth. The basal ration used in the first series was a general starter type ration with slight modifications in the second series. Choline chloride was added to the experimental diets at levels of 1%, 2% and 4% in the first series and 1% in the second series.

The results obtained, as summarized in Table I, show that with increasing levels of choline in the diet there is a retardation of the rate of growth. The addition of 1% choline brought about decreases in gains of 13.2% and 10.8% respectively in the 2 series. The addition of 2% and 4% of choline to the diet resulted in decrease in the rate of gain of 13.8% and 23.8% respectively as compared with the gains made on the basal ration.

The data in Table I have been treated statistically by taking the gains for the males and for the females in each lot. The significance of the difference in the mean gains between the males on the basal ration (lot 1) and the males in lot 2 receiving 1% of choline has been tested according to Fisher's t value. Likewise the t value for the significance of the means between the males in lot 1 and the males in lot 3, and the males in lot 4 have been calculated. Similarly the t values were calculated for the females between lot 1 and each of the other lots. The significance of the difference in mean gains for the females between lots 5 and 6, and for the males of the 2 lots, was also tested. The addition of 1% or more of choline to the basal ration produced a significant ($P = 0.05\%$) decrease in the rate of gain for the males, while in the case of the females the decrease in rate of gain was significant at the 2% level of choline and highly significant at the 4% level of choline in the diet. The decrease in rate of gain for the females in the 1% level of choline was not significant, but on the basis of the average t value for males and females on this level the difference becomes significant at this level in both series I and II.

Other than the reduced rate of gains no

gross pathological manifestations were observed as a result of the inclusion of amounts of choline chloride up to 4% of the ration.

Four representative specimens from each lot were slaughtered, and showed appreciable differences in body fat deposits. Carcasses of chickens fed the diet with 4% added choline had considerably less subcutaneous, abdominal and mesenteric fat than those fed the 1% and 2% levels, which in turn had less fat deposits than chickens receiving 0.50% of choline in the diet.

Summary. Various levels of choline chloride were added to rations fed to growing chickens. The addition of 1%, 2% and 4% reduced the rate of gain by about 12%, 13.8% and 23.8% respectively. As much as 4% of choline chloride added to the diet produced no toxic manifestations other than retarded growth.

15411

Ulnar-Femoral Nerve Anastomosis in Paraplegic Rhesus Monkey.*

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In seeking a method of bridging the gap in neural continuity caused by transection of the spinal cord, the following experiment was carried out.

The left ulnar nerve of a rhesus monkey was dissected from the axilla to the forearm and transposed subcutaneously to the left flank. The left femoral nerve was then exposed through a retro-peritoneal approach, and divided close to its origin where its proximal end was sutured to the distal end of the transposed ulnar nerve. For the sake of convenience, the left forelimb was then amputated at the shoulder.

Five and one-half months later the spinal cord was transected at D 4. Following transection it was evident that struggling movements resulted in active flexion of the left thigh despite the atrophy which had followed femoral nerve section.

Faradic stimulation of the anastomosed ulnar-femoral nerve through the intact skin, both above and below the point of nerve suture elicited contractions of the ipsilateral psoas and vastus muscles (innervated by the sutured femoral nerve).

Ten days after cord transection, the right motor cortex was exposed under ether anesthesia, and stimulated electrically. Stimulation of the exposed leg area, as expected, yielded no response. Stimulation of the forelimb area, however, with the same strength of current, elicited contractions of the contralateral (left) shoulder muscles and also simultaneous contractions of the left psoas and vastus muscles, innervated by the anastomosed femoral nerve. An ensuing generalized convulsive seizure involved not only the musculature above the level of the cord transection but also the above-mentioned muscles of the left thigh, which were the only muscles that participated in the convulsion below the level of cord transection. These movements were phasic and coordinated with the phasic movements of the upper extremities.

Electrical stimulation of the segments of the cervical cord giving rise to the transposed ulnar nerve yielded contractions of the ipsilateral thigh muscles while stimulation of the cervical cord elsewhere at the same current strength failed to do so.

On exposing the anastomosed ulnar-femoral nerve, it was found that direct electrical stimulation of the nerve both above and below the point of suture elicited flexion of the left thigh. There was no evidence that stimulation of the skin (or other structures) or of the anastomosed femoral nerve itself, distal to the suture line, caused a pain reaction. Microscopic studies of the anastomosed

* Aided by the William J. Matheson Commission.

nerves showed that regenerated nerve fibers had traversed the line of suture.

Conclusions. Clinical, electrical and microscopic studies demonstrated functional continuity after ulnar-femoral nerve anastomosis in a paraplegic rhesus monkey.

15412

Non-Utilization of Intravenously Administered Acetyl-*dl*-tryptophane.

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Albanese, Frankston, and Irby,[1] reporting on the fate of acetyl-*dl*-tryptophane given by mouth to human subjects, conclude "that, with the exception of a 5% urinary loss, all of the acetyl-*dl*-tryptophane may be available to man."

We have had occasion recently to investigate the fate of acetyl-*dl*-tryptophane administered intravenously to human subjects. Since the conclusions resulting from our investigation differ from those of Albanese *et al.*, publication of our findings is indicated: the difference in routes of administration must, of course, be emphasized.

Acetyl-*dl*-tryptophane (Merck) was used throughout. Two colorimetric methods for analysis of urine samples were employed. In the first of these the urine was diluted 100 fold. Portions of 1.0 cc were rendered strongly alkaline with 4.0 cc of 0.5 N NaOH and treated finally with 1.0 cc of the Folin-Ciocalteu phenol reagent.[2] The constituents were thoroughly and rapidly mixed and the resultant color read in a Klett-Summerson colorimeter after 2 minutes. The galvanometer deflections obtained were referred to a standard rectilinear reference plot of the corresponding readings obtained for varying quantities of 0.001 M acetyl-*dl*-tryptophane similarly treated with alkali and phenol reagent.

In the second method *p*-dimethylaminobenzaldehyde, as used by May and Rose[3] for tryptophane determination, was the reagent employed. Sodium nitrate, as proposed by Bates,[4] was used to accelerate the color development. Again the urine was diluted 100 fold. Portions of 0.25 or 0.50 cc, further diluted with water to 1.0 cc, were treated with 1.0 cc of sodium nitrate solution (1 mg per cc) and 5.0 cc of 0.05% *p*-dimethylaminobenzaldehyde in 12 N HCl. Readings were made on a Klett-Summerson colorimeter after 55 minutes and were corrected for the color given by normal urine. The reference curve, based on the use of 0.0 to 1.0 cc portions of 0.0005 M acetyltryptophane was rectilinear.

Recovery experiments on urine which was 0.0010 M to 0.0016 M with respect to added acetyltryptophane yielded a 96 to 101% recovery by either method. The correction for the color obtained with normal urine with the aldehyde reagent was, however, substantially lower than that obtained with the phenol reagent.

Three adult male subjects were used. In the first experiment each received intravenously 50 cc of 0.30 M acetyltryptophane adjusted to pH 7.2 with sodium hydroxide, passed through a sterilizing asbestos filter, and briefly heat-sterilized before use. The urine was collected in 3 post-injection periods of 6, 6 and 12 hours respectively, except in the third

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1 Albanese, A. A., Frankston, J. E., and Irby, V., *J. Biol. Chem.*, 1945, **160**, 31.

2 Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, 1927, **73**, 627.

3 May, E. E., and Rose, E. R., *J. Biol. Chem.*, 1922, **54**, 213.

4 Bates, R. W., Proc. Am. Soc. Biol. Chem., *J. Biol. Chem.*, 1937, vii, 119.

TABLE I.
Excretion of Acetyl-*dl*-tryptophane After Intravenous Administration.

Subject	Dose of acetyltryptophane	Acetyltryptophane excreted in 24 hrs in percentage of amount given*	
		Phenol method†	Aldehyde method
B	50 cc 0.3 M	80	79
L	"	83	83
H	"	74	78
B	50 cc 0.08 M		70
L	"		78

* Corrected for "apparent" tryptophane in pre-injection samples.

† Any tryptophane or tyrosine, and possibly other similar substances, in normal urine also give a blue color with the Folin-Ciocalteu reagent. However, the amount of color obtained under the above conditions with normal urine samples was small, and all values obtained were corrected for the colorimetric reading obtained with a normal 24-hour urine sample from the same subject. Both methods yield color with either tryptophane or acetyltryptophane. That the substance excreted by the experimental subjects was acetyltryptophane was evident since the amount present in the urines far exceeded the solubility of tryptophane, and in addition, acetyltryptophane could be readily isolated from the experimental urine samples.

subject where the collection periods were 3, 3 and 18 hours. Pre-injection 24-hour samples were analyzed for comparison.

In a second experiment, 2 of the above subjects received 50 cc portions of 0.08 M acetyltryptophane and urine was collected in 3 post-injection periods of 3 hours each. The purpose of this experiment was to determine whether the excretion of acetyltryptophane, observed in the first experiment to be a very high percentage of the amount given, was abundant only because of the comparatively large amount given. The results are presented in Table I.

Over 95% of the acetyltryptophane excreted was found to be voided within 6 hours of administration. Of this, the major portion was excreted within 3 hours.

Excretion of acetyltryptophane, in copious amounts, was confirmed by direct isolation. For this purpose the urine was acidified by addition of 1 cc of 6 N HCl per 10 cc of urine and allowed to stand at +1°C for 10 to 24 hours. The crystalline precipitate was centrifuged off and washed several times with cold water. It was dissolved in the minimum volume of hot ethyl alcohol, decolorized with charcoal and filtered. Four volumes of water were added to the filtrate and the solution placed in the cold room overnight for crystallization. The crystals were harvested again, decolorized, and twice recrystallized. From the urine of Subject B 1.7 g of final product were obtained, as compared with the 3.0 g estimated to be present on the basis of the colorimetric analysis. Since the isolation procedure was for qualitative rather than for quantitative purposes, it is clearly evident that the quantity of acetyltryptophane excreted must have been well in excess of the quantity of recrystallized product actually isolated. The substance possessed the chromogenic activity, crystal structure, and solubility of acetyltryptophane and was concluded to be such.

The rotation of a 10% solution of the substance was found to be +0.09°, that of the original acetyl-*dl*-tryptophane in 10% solution was +0.07°. The calculated rotation for a 10% solution of acetyl-*d*-tryptophane is —3.1°. We conclude, therefore, that acetyl-*dl*-tryptophane was excreted and no preferential utilization of acetyl-*l*-tryptophane is to be observed when the racemic compound is intravenously administered. We conclude further that acetyl-*dl*-tryptophane, given intravenously, is so rapidly excreted and so poorly utilized by the human subject that its incorporation in protein hydrolysates intended for intravenous alimentation is of little value. Likewise, the nutritive value of acetyl-*dl*-tryptophane in "stabilized" serum albumin appears to be negligible.

Summary. Acetyl-*dl*-tryptophane in aqueous solution was administered intravenously to

3 human subjects. Seventy to 83% of the administered substance was excreted unchanged, almost all within the first 6 hours after injection. The substance excreted was racemic: there was no preferential utilization or retention of acetyl-*l*-tryptophane.

The work described in this paper was done under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Stanford University.

15413

Effect of Quinacrine Hydrochloride (Atabrine) on Isolated Mammalian Heart.

L. H. SMITH AND J. D. STOECKLE. (Introduced by Otto Krayer).

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In laboratory animals (dog, cat, rabbit) the intravenous administration of atabrine causes a fall of blood pressure and, if the doses are large enough, disturbance of respiration.[1,2] As is the case with many substances, the toxicity is directly related to the rate of injection.[3] Studies on preparations of the isolated heart of the frog[4] as well as of mammals[1,5] suggest that atabrine has a negative inotropic action.

On the basis of the available clinical evidence[6–9] it appears possible that the negative inotropic action may play a rôle under certain clinical conditions when atabrine is injected intravenously in relatively large doses.

While the therapeutic levels, with the routine oral treatment now accepted, range between 25 and 75 μg per liter of plasma[10] and in total blood are of the order of 3 to 6 times these values;[9] in the experiments of Table VII in the paper of Shannon *et al.*, levels of the order of 1 mg per liter of whole blood and higher must have been present immediately after the rapid intravenous injection of the dose of 0.5 g.

Unna[2] ascribes the circulatory disturbance to action of atabrine upon the central nervous system. Before it is possible, however, to exclude the involvement of the heart in circulatory disturbances due to atabrine, it appears necessary to know at what atabrine blood level the heart begins to be affected. The following experiments on the heart-lung preparation of the dog were therefore undertaken to determine the minimum blood concentration of atabrine at which toxic effects are observed in the isolated mammalian heart.

It is fully appreciated that the atabrine concentration in whole blood does not represent the concentration in equilibrium with the body tissues because of the distribution of atabrine among the various components of the blood. It is our opinion, however, that our data are valid for the purpose of indicating the concentration range within which toxic effects upon the heart may be encountered, and that they apply to clinical

1 Hecht, G., *Arch. f. exp. Path. u. Pharmakol.*, 1933, **170**, 328.

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3 Dawson, W. T., Gingrich, W., and Hollar, E. D., *Am. J. Trop. Med.*, 1935, **15**, 515.

4 Suffolk and Berkshire, The Earl of, *Quart. J. Exp. Physiol.*, 1939, **29**, 1.

5 De Langen, C. D., and Storm, C. J., *Geneesk. tijdschr. v. Nederl.-Indië*, 1934, **74**, 1646.

6 Eckhardt, A. E., *Arch. f. Schiffs-u. Tropen-Hyg.*, 1933, **37**, 475.

7 Mayer, M., *Arch. f. Schiffs-u. Tropen-Hyg.*, 1933, **37**, 479.

8 Hiemann, H. L., and Shapiro, B. G., *Brit. Heart J.*, 1943, **5**, 131.

9 Shannon, J. A., Earle, D. P., Jr., Brodie, B. B., Taggart, J. V., Berliner, R. W., and Resident Staff of the Research Service, *J. Pharm. and Exp. Therap.*, 1944, **81**, 307.

10 Ellerbrook, L. D., Lippincott, S. W., Cateno, C. F., Gordon, H. H., and Marble, A., *Arch. Int. Med.*, 1945, **76**, 352.

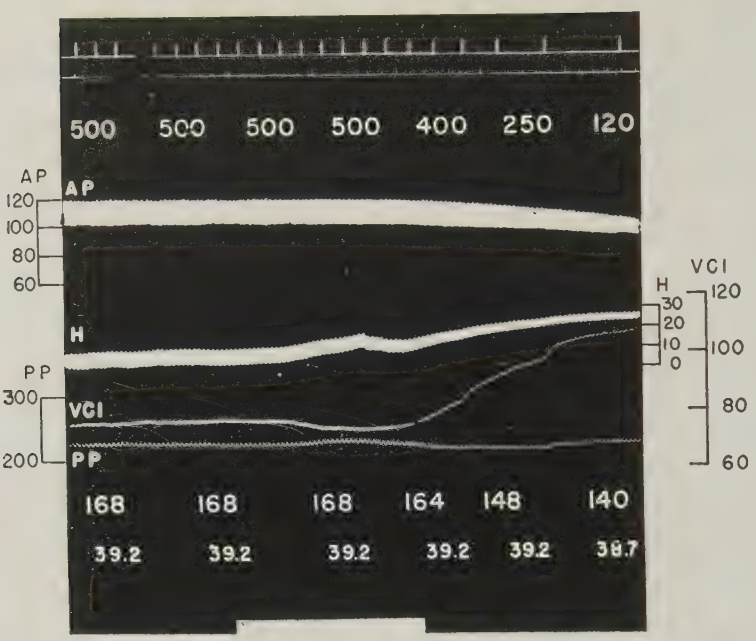

FIG. 1.

The effect of atabrine upon the isolated mammalian heart. Dog. Female. 8.2 kg. Heart-lung preparation. Arterial resistance, 78 mm Hg. Weight of ventricles, 49 g. Tracings indicate from above downward systemic output in 100 cc; time in 10-second intervals; arterial pressure (AP) in mm Hg; heart volume (H) in cc; right atrial pressure (VIC) measured from inferior vena cava in mm water. Pulmonary pressure (PP) in mm water recorded with a Bromoform manometer from a branch of the right pulmonary artery. The horizontal rows of figures indicate from above downward; systemic output in cc per minute; heart rate per minute; temperature of blood in degrees centigrade. During the period indicated by the signal 20 mg of atabrine were injected. The total blood volume was 830 cc.

conditions, as it is not unlikely that the human heart is more sensitive than the canine heart.[9]

Methods. Twelve heart-lung preparations were made according to Patterson and Starling.[11] The arrangement used was described in detail by Krayer and Mendez.[12] The dogs weighed between 9 and 12 kg. They were anesthetized with sodium pentobarbital 35 mg per kilo body weight injected intraperitoneally. Defibrinated blood was used. The total blood volume at the beginning of the experiments was between 600 and 900 cc. Arterial resistance was about 75-85 mm Hg to ensure a mean blood pressure between 100 and 120 mm Hg at a basal systemic output of 400-500 cc. Recordings were taken in all experiments of arterial pressure, right auricular pressure, pulmonary arterial pressure, heart rate, systemic output (= total output of left ventricle minus coronary flow). In some of the experiments heart volume, coronary sinus outflow, and electrocardiogram were followed. The atabrine was used in the form of the dihydrochloride. The doses and concentrations given refer to the salt.

To insure uniform distribution, fresh solutions of atabrine dihydrochloride were added to the blood before it entered the venous reservoir. Injections were made in single or divided doses, or by continuous infusion. Samples of blood were taken at intervals throughout the experiment and analyzed for atabrine, using the double extraction method of Brodie and Udenfriend.[13] Checks carried

[11] Patterson, S. W., and Starling, E. H., *J. Physiol.*, 1913, **48**, 357.

[12] Krayer, O., and Mendez, R., *J. Pharm. and Exp. Therap.*, 1944, **81**, 307.

[13] Brodie, B. B., and Udenfriend, S., *J. Biol. Chem.*, 1943, **151**, 299.

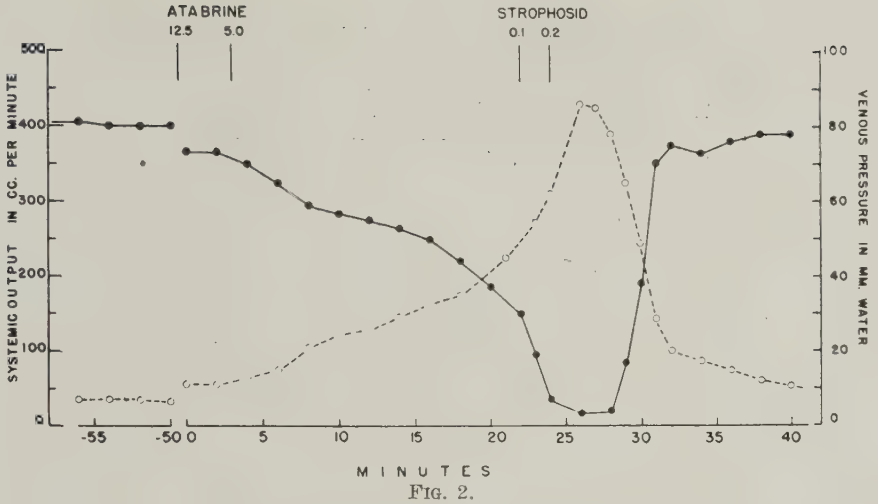

FIG. 2.

Effect of a cardiac glycoside on the myocardial failure caused by atabrine. Dog. Male. 9.7 kg. Heart-lung preparation. Arterial resistance, 78 mm Hg. Weight of ventricles, 75 g. —— ● —— output in cc per minute, – – ○ – – right atrial pressure in mm water. The dosage figures of atabrine and strophosid indicate mg. (For further details see Exp. 6, Table I).

out with known amounts of atabrine added to defibrinated blood to determine the accuracy of the method gave recovery values of 90% or better.

Results. I. *The negative inotropic cardiac action of atabrine.* The characteristic effect of atabrine in the heart-lung preparation is an impairment of contractility leading to a decrease in output. This is illustrated by Fig. 1. In this experiment 20 mg of atabrine hydrochloride were administered by slow injection within 2 minutes. At the end of the injection the systemic output decreased to 80% of normal and within another 2 minutes fell to 24% of normal. At the same time there was a gradual increase in right atrial pressure and a marked increase in the volume of the heart. As there was no significant change in pulmonary pressure throughout the period of this change, the increase in volume of the heart must be ascribed largely to a negative inotropic action of atabrine. The decrease of the stroke volume of the heart can be seen from the width of the heart volume curve and is very marked in spite of the drop in heart rate to 83% of the normal rate.

As in all cases of heart failure occurring with this method of investigation, the increase in right atrial pressure was a good indicator of the onset, the development, and the intensity of the failure. The pulmonary arterial pressure changes were not consistent. In some experiments the pressure fell, in others it was not changed (as in Fig. 1), while in some it rose. Coronary sinus outflow measured in several experiments did not change significantly. The decrease in the systemic output of the heart, therefore, reflects the decrease in the output of the left ventricle and not a shunting of the blood through dilated coronary vessels as it may occur in spontaneous heart failure.

Bradycardia was not a constant finding. In 5 experiments the rate decreased by from 6 to 32 beats per minute. There was no correlation between degree of bradycardia and severity of the negative inotropic effect. In experiments without irregularities of rhythm the electrocardiogram did not reveal any characteristic differences from the normal records. In 4 experiments irregularities of rhythm occurred which seemed to be due to extrasystoles, but no electrocardiographic analysis was made in these experiments.

In no instance was the negative inotropic action or the disturbance of rhythm spontaneously reversed. In confirmation of ob-

TABLE I.
Concentration of Atabrine in the Blood of Heart-Lung Preparation of the Dog After Single and Divided Doses of Atabrine.

Exp. No.	Time, min.	Atabrine inj., mg	Atabrine conc., mg/L blood	Blood vol., cc	Wt of Heart	Remarks
2	0	15.0		785	80	
	2.5		5.9			Rapid rise of right atrial pressure, severe failure.
	11.0		4.1			
	17.4		3.1			
3	0	12.0		600	70	Marked irregularities of rhythm, rise of
	2.8		4.1			right atrial pressure.
4	0	10.0		780	65	
	2.5		1.4			Slow rise of right atrial pressure, gradual development of failure.
	9.0		1.0			
5	0	5.0		690	75	No effect.
	6.5	5.0		620		Slow rise of right atrial pressure,
	14.5		1.9			gradual development of failure.
	24.2	5.0		500		
	26.7		4.9			Severe failure.
6	0	5.0		875	75	
	11.2	5.0		860		No effect.
	15.0		1.4			,, ,,
	22.5		1.0			,, ,,
	27.2	2.5		810		
	30.7		2.0			Gradual rise of right atrial pressure.
	44.2	5.0		765		
	47.2		3.1			Severe failure.
	52.7		2.6			
	61.7		2.1			(See Fig. 2).
7	0	5.0		840	91	
	8.3		0.2			No effect.
	12.5	5.0		775		
	16.0		1.0			Slow rise of right atrial pressure, gradual development of failure.
	23.5		0.8			

servations of De Langen and Storm it was found that epinephrine hydrochloride readily restored an increased work capacity to the failing heart. The effect, however, was transient. Cardiac glycosides, on the other hand, had a long-lasting improving effect upon contractility. Ouabain and K-strophosid were each used in several experiments. Fig. 2 illustrates the effect of K-strophosid. The restoration of the work capacity of the heart is seen to be virtually complete. There was no significant change in heart rate in this experiment. Even if the heart failure occurred much more acutely than in the experiment of Fig. 2, the cardiac glycosides were able to effect a complete reversal.

II. *The minimal toxic blood concentration of atabrine.* Blood levels were determined in 7 of our experiments. The results are shown in Table I and Table II. The minimal toxic blood concentration is approximately 1 mg per liter. Concentrations below this were not found to have an effect; above 1.5 mg per liter negative inotropic effects were found to occur regularly. Large single doses caused severe toxic effects very quickly when injected rapidly, while the same dose given over a period of 2 minutes (see Fig. 1) led to negative inotropic effects which developed more gradually and as a rule were less severe. By slow administration relatively large amounts could be introduced without leading to toxic

TABLE II.

Concentration of Atabrine in the Blood of the Heart-Lung Preparation of the Dog During Constant Infusion. (Exp. 8). Dog, male, 9.5 kg. Arterial resistance, 70 mm Hg. Heart weight at end of experiment, 75 g. Infusion rate at beginning, 0.5 mg per minute, from minute 53 on, 1 mg per minute.

Time, min.	Concentration mg/L blood	Total blood vol., cc	Remarks
0	—	705	Infusion starts.
17	1.1	680	No effect.
28	1.2	650	" "
37	—		Right atrial pressure begins to rise.
40	1.7	620	Right atrial pressure continues to rise gradually.
50	2.0	570	Some decrease in competence.
53	—		Rate of infusion doubled.
58	4.1	510	Marked increase in right atrial pressure. Rapid development of myocardial failure.
62	6.1	470	

effects as in the experiment of Table II. In this experiment atabrine was administered during the first 50 minutes at the rate of 0.5 mg per minute. At 28 minutes when 14 mg had been injected, the blood level was 1.2 mg per liter and no negative inotropic effects were noticeable. These started at 37 minutes at a blood concentration between 1.2 and 1.7 mg per liter, which is in good agreement with the results presented in Table I.

III. *The disappearance of atabrine from the blood.* From experiment 2 of Table I it is evident that the atabrine concentration in blood decreased by 50% in 15 minutes. The trend of this decrease is obvious in several of the other experiments. Similarly, from the experiment of Table II it follows that atabrine is removed from the blood almost as rapidly as it is infused. After 50 minutes when 25 mg of atabrine had been infused at a rate of 0.5 mg per minute the concentration in the blood was 2.0 mg per liter. This means only 1.1 mg, or 4.5% of the amount administered, remained in the total blood volume of 550 cc. When the infusion rate was at this time increased to 1.0 mg per minute, a rapid rise in blood level from about 2 mg per liter to 6 mg per liter occurred within 10 minutes, corresponding to a retention in the blood of about 20% of the atabrine administered during this period.

Discussion. While Unna concluded from his experiments in intact cats that the toxic circulatory action is to be attributed to a central vasomotor depression occurring concomitant with respiratory stoppage, our experiments on the dog heart prove, in confirmation of observations of Hecht[1] and De Langen and Storm,[2] that atabrine has a negative inotropic action on the isolated heart. Furthermore, severe myocardial impairment can occur with no or only a slight decrease in rate and no marked changes in the electrocardiogram.

The minimal negative inotropic concentration of 1 mg per liter of blood admittedly is far above the therapeutic blood concentration required in human malaria. With appropriate doses given orally, levels of 1 mg per liter of blood are not reached. There can be no doubt, however, that intravenous injections rapidly performed may increase to dangerous levels the atabrine concentration reaching the coronary circulation, as evidenced by the figures of whole blood concentrations in the experiments of Shannon *et al.* in man quoted above.[9]

The experiment of Table II indicates the rate at which administered atabrine is removed from the blood, although in the heart-lung preparation the heart itself and the lungs are the only organs to take up the drug. The rapid rise of blood concentration when at minute 53 the rate of infusion is doubled suggests 2 probable explanations: either the tissues may have been nearly saturated by the infusion of approximately 25 mg and the rate of removal would have dropped off even with the low rate of administration; or heart and lungs remove atabrine from the blood at a limited rate and with an increase in rate

of administration above 0.5 mg per minute concentration in the blood rises steeply because the rate of removal does not correspondingly increase. This question needs further study.

A comparison of the results of Table I and Table II explains why injudiciously fast intravenous administration of atabrine may readily lead to toxic effects upon the heart muscle in the isolated heart, not encountered when even large amounts are given by slow intravenous administration, and entirely unlikely in the intact animal or man when appropriate therapeutic doses are given orally.

Summary. In the isolated heart of the dog in the form of the heart-lung preparation atabrine has a negative inotropic action which appears in some cases at concentrations of 1 mg per liter of blood and is always observed at concentrations of 1.5 mg per liter or above. As a result, the work capacity of the heart is impaired. Slowing of the heart rate sometimes occurs. Irregularities of cardiac rhythm are seen only with high and acutely toxic doses.

Injection of 10-15 mg of atabrine within 1 minute into a total blood volume of 500 to 900 cc leading abruptly to blood levels of several milligrams per liter of blood causes a severe heart failure, while the continuous infusion of the same amount at a rate of 0.5 mg per minute will barely reach the toxic concentration in the blood.

A severe myocardial failure of the isolated heart, once established, does not appear to be reversible. Epinephrine hydrochloride has a transient positive inotropic and chronotropic action, while the cardiac glycosides effect a prompt and long-lasting improvement of the work capacity of the failing heart in doses which do not change the heart rate and do not lead to irregularities of heart rhythm.

15414

Control of pH in Roller Tube Cultures.

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The purpose of these experiments was to determine in roller tube cultures the pH range of media which would support growth of fibroblasts. The study was undertaken as preliminary to the cultivation of bone *in vitro* under standardized conditions. It was prompted by the fact that the optimal reaction for the activity of alkaline bone phosphatase lies between pH 8 and pH 10.[1] Such values are distinctly beyond the range of pH considered desirable for tissue *in vitro*. In the case of chick tissue the accepted pH range is 7.2 to 7.8.

Various methods of controlling the pH range suggested themselves. The obvious was either to choose buffers operative in this alkaline range (8 to 10) or to use the ordinary solutions, such as Tyrodes, and control the amount of CO_2 in equilibration with the media. We chose the latter approach and devised an apparatus for continuous introduction of the gas.

Technic. The apparatus used to introduce gases into the roller tubes has been described.[2] The following gases were introduced into the tubes: air without CO_2; air; air with 1%, 2%, 4%, 8%, 16% and 32% CO_2. Air with no CO_2 was made by first drawing approximately 18 liters of air into a 5-gallon bottle and introducing a 10% NaOH wash bottle in the pipe line to the roller tubes. Air alone required no special treatment. All of the CO_2 mixtures were made with the aid of a Kipp generator. The gas displaced the proper amount of water and by siphoning

[1] Williams, H. L., *U. Western Ont. Med. J.*, 1940.

[2] Paff, G. H., and Samuelsen, G., Proc. Soc. Exp. Biol. and Med., 1946, **62**,

TABLE I.
Hydrogen Ion Concentration of the Fluid Media at the Beginning and End of Air Flow Experiments.

Experiment	Number of cell colonies	pH of media at start	pH of media at end
CO_2 free air	40	7.57	9.17
Controls	40	7.57	7.63
Air alone	120	7.64	8.36
Controls	120	7.64	7.65
1% CO_2 in air	40	7.55	7.60
Controls	40	7.55	7.80
2% CO_2 in air	40	7.60	7.38
Controls	40	7.60	7.70
4% CO_2 in air	40	7.58	7.32
Controls	40	7.58	7.75
8% CO_2 in air	40	7.60	6.9
Controls	40	7.60	7.74
16% CO_2 in air	40	7.55	6.75
Controls	40	7.55	7.60
32% CO_2 in air	40	7.50	6.55
Controls	40	7.50	7.62

of additional water, the necessary amount of air was drawn into the bottle. Since CO_2 is highly soluble in H_2O the precaution was taken of adding 2 to 3 cc of concentrated H_2SO_4 to the water in contact with the gas.

All experiments were conducted over a 2-day period and the amount of gas delivered to each tube was 2 liters per day. For tests with each gas at least 4 roller tubes each containing 10 cultures were prepared. Controls were run simultaneously in the form of 4 completely sealed tubes each containing 10 cultures. All tests employed fibroblasts derived from the tips of ventricles from the hearts of 8-9-day chick embryos. A ventricle was excised and ½ to 1 mm cubes were cut. Care was taken to apportion fragments impartially between experimental and control tubes. After the fragments were set in the plasma lining the tube and clotting had occurred, each tube received 3 cc of 25% chick embryo extract of known pH.

At the end of 48 hours mineral oil was layered on the surface of the liquid media in the experimental tubes. This was done as the gas flowed through the tube. The fluid medium was then removed and a pH determination was made under oil using a Beckman pH meter. After determining the pH of both the experimental and control culture media the cultures were fixed with 10% formaldehyde. The amount of growth was determined by projection outline drawings of the cultures at a magnification of 55 ×. Both the original culture and the peripheral limits of growth (and migration) were outlined. The area of growth was computed by the paper weight technic. Controls were considered as showing 100% growth. The growth of the experimental cultures was figured in % relationship to the controls. Exclusive of many preliminary experiments our data is based on 800 colonies of fibroblasts.

Observations. Table I gives a list of the pH values for the liquid media at the start and after 48 hours for each experiment. It will be noticed that the pH of all media at the start was practically the same for all experiments. It ranged from 7.5 to 7.64. After 48 hours of cultivation the expected spread in pH became evident. It ranged from the noticeably acid 6.55 of the 32% CO_2 in air to the distinctly alkaline pH of 9.17 for CO_2 free air.

In Fig. 1 the amount of growth of each of the "air flow" tubes is represented. It

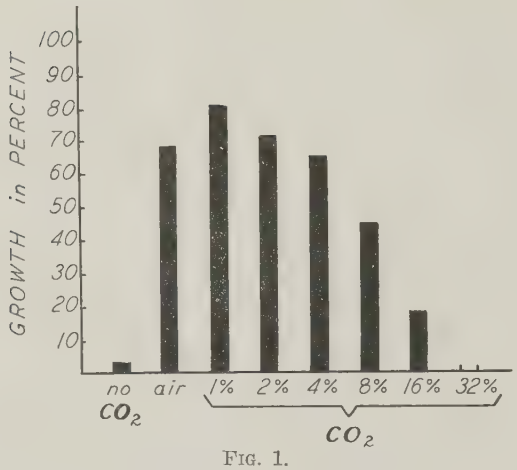

Fig. 1.
Graphic representation of extent of growth in air flow tubes. For explanation see text.

can be seen that in flow tubes the greatest amount of growth occurred with air, and 1%, 2% and 4% CO_2 in air. Only 3.4% growth was obtained with CO_2 free air. No growth occurred in 32% CO_2 in air.

The only obvious morphological difference observed among the cultures of different ionic concentration was that toward the acid side the cells showed more vacuoles than they did on the basic side. Fischer[3] made the same observation and Bauer[4] observed additional effects in drop cultures treated with high CO_2 tensions.

Discussion. These results indicate the range of pH possible in roller tube cultures by the simple procedure of changing the CO_2 content of the atmosphere. The technic has proven to be much simpler than making a series of solutions of varying pH. It is hoped that this method may lead to more studies of various tissues cultivated at different values of pH. Based solely on fibroblast studies the idea has become fixed that in tissue culture studies the media should have a pH in a rather fixed range. For chick fibroblasts obtained from certain sources this range (7.2 to 7.8) is optimal. One might well wonder if the same range of pH is optimal for gastric epithelium, or the deep lying cells in hyaline cartilage, or for renal epithelium. The blood pH is fixed within narrow limits but local economy in many parts of the body must operate under hydrogen ion concentrations well removed from that of the blood.

As to the results themselves, it will be noticed in Fig. 1 that growth is expressed in %. The amount of growth in the controls run simultaneously with experimental cultures was considered to be 100%. None of the values in the "air flow" tubes reached this value. The nearest approach to the large area of growth of control cultures was in the 1% CO_2 in air cultures. Here the growth was 81%. To go further, in our air flow experiments the optimal pH range based on extent of growth lay between 7.32 (pH of 4% CO_2 in air cultures) and 8.36 (pH of air alone cultures). This range is at variance with the findings of Fischer,[4] with solid media. On the basis of careful work Fischer found that optimal growth occurred between pH 7.0 and 7.8. We obtained very good growth in flow tubes in which the atmosphere was untreated air. This was not expected because of the relatively high alkalinity. The average of 12 pH determinations (all of which gave values above pH 8.0) was pH 8.36. There is no doubt that growth was good. The experiment with untreated air was repeated with comparable results. The average growth of 120 cell colonies was 69% of the control values. The control cultures themselves grew luxuriantly.

In drop cultures Lewis and Felton[5] obtained abundant growth in media having a hydrogen ion concentration from pH 6.0 to pH 9.0. These limits are broader than those obtained in roller tubes but the difference may be more apparent than real. We worked with CO_2 as our controlling instrument whereas Lewis and Felton (Fischer also) controlled their pH with media of variable composition. It is not easy to prevent drifts in pH determinations of solutions whose CO_2 tensions differ markedly from the atmosphere above them. If drifts occurred they were toward the pH value of the media existing

[3] Fischer, A., *Tissue Culture,* Levin and Munksgaard, Copenhagen, 1925, 71.

[4] Bauer, J. T., *Bull. J. Hopkins Hosp.,* 1925, **37**, 420.

[5] Lewis, M. R., and Felton, L. D., *Science, N. S.,* 1921, **54**, 636.

before CO_2 was added. We made our pH determinations under oil but our lower limit of 6.55 could well have been 6.3 or even lower. At the other extreme our 8.36 value could have been higher. Indeed, among the 12 determinations of which 8.36 is the average, there were readings of 8.5 and 8.6.

The pH of the control media became slightly more alkaline by the end of 48 hours (Table I). One might expect tissue metabolites to cause a shift toward the acid side. In roller tubes with 3 cc of a buffered fluid medium above small fragments of tissue growing in a thin lining of blood plasma, the acidifying effect of metabolites is negligible. The alkaline shift in the control media may have been due to the presence of alkali in the glass.

Summary and Conclusions. Employing a flowing atmosphere in roller tube cultures the range of hydrogen ion concentration permitting growth of fibroblasts was ascertained. The atmosphere was varied to produce the pH desired. The gases with the resulting pH of the media were as follows: air with no CO_2, pH 9.17; untreated air, pH 8.36; air with 1% CO_2, pH 7.6; with 2% CO_2, 7.38; with 4% CO_2, 7.32; with 8% CO_2, 6.9; with 16% CO_2, 6.75; and with 32% CO_2, pH 6.55. The best growth was obtained with untreated air and with air containing 1%, 2%, and 4% CO_2.

15415

An Apparatus for Permitting Control of the Atmosphere in Roller Tube Cultures.

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Various types of containers have been devised to permit control of the atmosphere overlying tissue cultivated in hanging drops[1] and in stationary flasks[2,3] *in vitro*. None of these are easily adaptable to roller tube cultures because of the constant rotation of the drum in which the tubes are placed. Ott, Tennant, and Liebow[4] suggested that a controlled atmosphere can be provided for roller tube cultures if a Novy jar is mounted on the drum. The difficulties and limitations of such a procedure are obvious.

Recently we have found it necessary to devise an efficient apparatus which would permit complete control of a flowing atmosphere at all times and eliminate both the handling of the tubes and the opening of the incubator. This apparatus has given us such satisfactory results that it seems worthy of description.

In order to insure a constant flow of air (or any desired gas) through the roller tubes, the gas is first made to displace water from a reservoir, such as a 5-gallon bottle. After all of the water is displaced, a fine steady stream of water is permitted to flow into the gas-filled bottle to displace the gas. The gas is then led into the incubator where it passes through the apparatus shown in Fig. 1. The gas enters tube "A" and passes successively through tubes "B," "C," "D" and "E" and then to the roller tubes "F." Tube "A" is fixed, tube "B" is movable and fits over tube "A": the mercury seal prevents the leakage of gas. "C" is a rubber tube which allows the movement of the horizontal shaft "D" to be transmitted to the vertical tube "B." Tube "D" is a part of the drum axle which is hollowed out to permit the gas to

[1] Bauer, J. T., *Bull. Johns Hopkins Hosp.*, 1925, **37**, 420.

[2] Parker, R. T., *Medical Physics*, Year Book Publishers, Inc., 1944, 1578.

[3] Carrel, A., *Compt. rend. Soc. biol.*, 1934, **117**, 1144.

[4] Ott, L. H., Tennant, R., and Liebow, A. A., *Science*, 1940, **91**, 437.

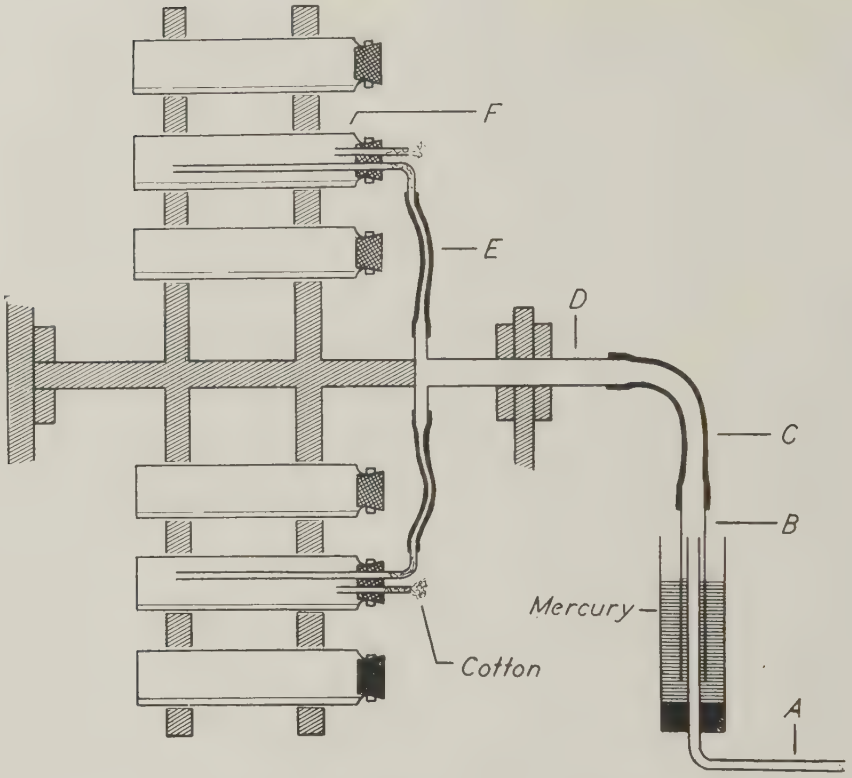

FIG. 1.
Sectional view of apparatus permitting control of the atmosphere in roller tube cultures. No attempt was made to diagram the driving mechanism of the drum. For description of remainder of apparatus see text.

flow through tubes "E" to the roller tubes "F" (in the sectional view of the apparatus only 2 "E" tubes are shown although 4 were used). The gas is led through a long fine bore tube to the bottom of the roller tube (this long tube extends into the roller tube only far enough to clear the fluid medium when the tube is set upright). From the roller tube the gas is allowed to escape into the atmosphere of the incubator. Small loose cotton filters are sufficient to prevent air borne contamination.

This apparatus has been used now with several hundred cultures in roller tubes and it has been found to have the following advantages: (1) the composition of the atmosphere above the cultures can be controlled; graded concentrations of CO_2 in air have been used thus far but other gases or mixtures of gases can be tested for effects on various types of tissue; (2) control of the pH of the media is excellent since there is good equilibration between atmosphere and media; (3) mechanically the apparatus is easy to operate.

Although no special effort has been made to determine the optimal volume of "air flow" in the tubes we have had good results with a daily volume of 2 liters of atmosphere per tube.

15416

The Determination of Total Circulating Serum Proteins and Erythrocyte Volumes in Normal and Protein Depleted Rats.*

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(Introduced by Paul R. Cannon).

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In order correctly to evaluate the influence of variations in quality and quantity of ingested protein on the various blood components of an animal it is necessary to measure total quantities of circulating blood and plasma. Over a period of 2 years, during investigations on protein metabolism in this laboratory, we have performed approximately 700 blood volume estimations in rats. A method has been employed which differs sufficiently from other published methods to merit description. Previously used methods have been reviewed in detail by Metcoff and Favour.[1]

The use of diluted whole blood samples for estimation of dye concentration in blood volume determination is simpler and more economical of blood than the use of separated plasma or serum. One can use diluted whole blood for the dye determination, provided the dye concentration is high enough for accurate estimation, the volume of cells does not interfere with colorimetry, there is no significant hemolysis and the color of the dye is not altered by dilution.

With the method described, total blood volume may be determined on only 0.1 ml of blood, and plasma volume, red cell volumes, and hemoglobin on one sample of 0.2 to 0.3 ml of blood.

Methods and Materials. Adult male albino rats of the Sprague-Dawley strain weighing between 180 and 245 g were used. They were divided into 2 groups. One group was fed a ration containing 18% casein, the other a diet containing 1.8% protein (N $\times$ 6.25) derived mainly from carrots. The diets were isocaloric and offered in equal quantity each day. These diets and the method of depletion have been described in detail.[2]

At the end of 9 to 11 weeks on the diets, the animals were fasted overnight and the following determinations were then done: total blood volume, hematocrit, hemoglobin, and serum protein.

Dye Injection and Sampling. Blood volumes were determined using the dye T-1824. Under light ether anesthesia, using a calibrated tuberculin syringe and a ¾″ 24-gauge needle, one mg (0.30 ml of a 0.333% solution) of the dye was injected into a tail vein. The needle was held in place for approximately 15 seconds until dye had left the vein. Light pressure was applied over the point of injection, the needle was withdrawn, and pressure was maintained until bleeding ceased (usually within one minute). Four minutes following injection anesthesia was again begun and at 5 minutes $\pm$ 15 seconds, the animal was bled from a tail vein other than the one previously used for injection. The method of bleeding the rats has been described in detail.[3] Blood was collected directly from the tail into a 0.1 ml blood sugar pipette. Then 0.2 ml was collected in a small vial containing dry oxalate, (2.4 mg of ammonium oxalate and 1.6 mg of potassium oxalate). Finally, 0.5 ml was collected without anticoagulant for serum protein determination by the falling drop method.[4]

* This work was aided by grants from the John and Mary R. Markle Foundation, The National Livestock and Meat Board, The Douglas Smith Foundation of The University of Chicago, and Allen B. Wrisley and Co.

1 Metcoff, J., and Favour, C. B., *Am. J. Physiol.*, 1944, **141**, 695.

2 Wissler, R. W., Woolridge, R. L., Steffee, C. H., and Cannon, P. R., *J. Immunol.*, 1946, **52**, 267.

3 Cannon, P. R., Humphreys, E. M., Wissler, R. W., and Frazier, L. E., *J. Clin. Invest.*, 1944, **23**, 601.

4 Barbour, H. G., and Hamilton, W. F., *J. Biol. Chem.*, 1926, **69**, 625.

Dilution of Samples. The 0.1 ml of blood in the sugar pipette was introduced immediately after withdrawal into 2.0 ml of a special diluting fluid contained in a colorimeter tube. The diluted blood was mixed, then centrifuged 10 minutes at 1500 r.p.m. in an ordinary centrifuge. The special diluting fluid was composed of 2.0 g of purified beef plasma albumin† dissolved by stirring into 100 ml of 0.86% NaCl solution containing .06% of ammonium oxalate and .04% of potassium oxalate. This solution was centrifuged 15 minutes at 4000 r.p.m. in an angle centrifuge. The decanted supernate was pale yellow and clear, and could be kept for 24 hours in the refrigerator.

Colorimetry. Readings were made in a Klett-Summerson colorimeter using a 620 mμ filter. The colorimeter has been adapted by us to take standard Wassermann tubes utilizing a 2 ml volume. For blank controls, blood from 3 depleted and 3 normal fasted rats was treated as described for the dye-stained samples. The average of the 6 values was used in the calculation of dye intensity.

With each set of determinations a standard was run. It was set up as follows: Three-tenths ml of the T-1824 solution used in the animals was injected from the tuberculin syringe, needle attached, into 10.0 ml of the albumin diluent. One-tenth ml of this was then introduced into 2.0 ml of diluent in a colorimeter tube. This and its blank, the diluting fluid, were read in the colorimeter along with the unknowns, and the K value was calculated. In 16 duplicate measurements the coefficient of variation of K was ±0.66%.

Hematocrit and Hemoglobin Determinations. Hematocrit values were determined in thick walled capillary tubes having a bore of 1 mm and a length of 10 cm. An approximately 8 cm column of blood was drawn into the tube. One end, sealed with plasticene, was seated in the same material in an empty .38 caliber revolver cartridge. They were centrifuged at 2000 r.p.m. with an effective head radius of 25 cm for 30 minutes. The lengths of the total blood column and the red cell column were measured with a millimeter rule and the hematocrit was calculated.

Hemoglobin was determined in the Dick-Stevens photoelectric hemoglobinometer,[5] which requires approximately .02 ml of oxalated blood per determination. A correction was applied for the effect of the dye in the samples.

Calculations. From the estimated dye concentration and the K value for the dye standard the total blood volume was calculated. Consideration was taken of the fact that the 2.1 ml volume in the colorimeter tube was composed of approximately .04 ml of cells which did not enter into the volume of the dye-containing fluid.[5] Therefore, the actual volume was 2.06 ml. We have usually disregarded the ±0.01 ml variation which would be caused by a 10% deviation in the hematocrit. The largest error thereby introduced was no more than ±0.5%.

Plasma and erythrocyte volumes were calculated from total blood volume and hematocrit values.

Repeated Determinations. Repeated blood volume determinations can be made on the same animal. If the interval is less than a week it is necessary to correct for residual circulating dye. This requires a preinjection bleeding of 0.1 cc which is diluted and read in the colorimeter. This value is substituted for that of the blank of diluted whole blood described above.

Discussion and Results. Evans blue is relatively non-toxic in doses of 20 mg or less per kg of rat.[6] The quantity of dye used here was less than 10 mg per kg and we have observed no untoward effects in animals so treated.

Injection of the dye via tail vein eliminates operative procedures used in other methods.[1] This can be accomplished after a little practice without significant loss of dye. The tuberculin syringe should have a clear glass plunger; if not, a white line placed on the tip of the plunger helps in setting.

A major problem in any dye dilution method is time of sampling in relation to complete-

† Kindly supplied by Armour and Co.

[5] Dick, G. F., and Stevens, D. S., *J. A. M. A.*, 1940, **114**, 1065.

[6] Gibson, J. G., and Gregersen, M. I., *Am. J. Physiol.* (proc.), 1935, **113**, 667.

TABLE I.
Comparison of Various Blood Components in Normal and Depleted Rats.*

	Controls	Depleted
Body wt g	303 ± 7.7	155 ±1.2
Surface area cm² [16]	386 ± 5.0	258 ±1.3
Total blood vol. ml	18.7 ± 0.33	10.1 ±0.12
" erythrocyte vol. ml	8.6 ± 0.21	4.4 ±0.091
" plasma vol. ml	10.1 ± 0.46	5.8 ±0.094
Serum protein g %	6.22± 0.57	4.07±0.075
Total circulating serum protein mg	630 ±12.2	235 ±5.3
Hemoglobin conc.	15.7 ± 0.11	13.4 ±0.19
Hematocrit %	45.8 ± 0.46	43.3 ±0.74
Mean corpuscular hemoglobin conc. %	34.3 ± 0.28	31.3 ±0.43
Unit blood vol. $\frac{\text{ml}}{100\ \text{cm}^2}$	4.8 ± 0.063	3.9 ±0.042
" erythrocyte vol. "	2.2 ± 0.044	1.7 ±0.11
" plasma vol. "	2.6 ± 0.046	2.2 ±0.037
" serum protein $\frac{\text{mg}}{100\ \text{cm}^2}$	163 ± 2.8	91 ±2.1

* The values given are the means and following each the standard error. There were 23 animals in the control group and 30 in the depleted group.

ness of mixing. It has been shown[1] that between 3 and 5 minutes after the injection of Evans blue into a rat one obtains the most constant relationship between dye concentration and surface area. This has been interpreted as indicating time of complete mixing. Serial determinations of dye concentration in several rats showed that it reached a maximum at approximately 3 minutes after injection, remained constant to within 2% up to 7 minutes, then began to decrease, 8% having disappeared in 30 minutes. It has been shown that in men and dogs the single sample modification of the dye dilution technic using T-1824 agrees well with the carbon monoxide method,[7] and in men with the multiple sample extrapolation technic.[8] Sampling 5 minutes following dye injection in the rat appears to give adequate time of mixing. The error introduced, assuming a linear loss and 8% disappearance in 30 minutes, is less than 1%. A variation in bleeding time of even ±1 minute has introduced a negligible error.

The type of anticoagulant used may interfere with the determination of hemoglobin and dye concentration. Heparin even in small amounts imparts sufficient color to the plasma to interfere with the estimations.[9] Oxalate can be used, preferably as a mixture of potassium and ammonium salts. The concentration needed to prevent coagulation is much greater than with human blood.

T-1824 is bound largely to the serum albumin and at low concentrations of albumin the light absorption of the diluted dye is markedly depressed.[10,11] The rat's serum albumin concentration may vary from 2 to 4 g %.[12] If diluted 1:21 with saline for dye determination, the values may range from 0.07 to 0.14 g %. From Rawson's data[10] one can compute that such a variation in albumin concentration at a dye concentration of 4γ/ml may produce a variation in light absorption of as much as 8%. We have found upon actual experiment that when the rat serum albumin concentration was increased from .06 to .12 g % at a T-1824 concentration of 4 γ/ml there was a decrease in light absorption of 5.8%. This effect can be obviated by maintaining a sufficiently high

[7] Hopper, J., Jr., Tabor, H., and Winkler, A. W., *J. Clin. Invest.*, 1944, **23**, 628.

[8] Noble, R., and Gregersen, M. I., *J. Clin. Invest.*, 1946, **25**, 172.

[9] Gregersen, M. I., and Schiro, H., *Am. J. Physiol.*, 1938, **121**, 284.

[10] Rawson, R. A., *Am. J. Physiol.*, 1943, **138**, 708.

[11] Gregersen, M. I., and Gibson, J. G., *Am. J. Physiol.*, 1937, **120**, 494.

[12] Cannon, P. R., Wissler, R. W., Woolridge, R. L., and Benditt, E. P., *Ann. of Surg.*, 1944, **120**, 514.

TABLE II.
Blood and Plasma Volumes of Adult Normal Rats by Methods Utilizing T-1824.

Author	Plasma vol. ml/100 cm²	Total blood vol. ml/100 cm²
Beckwith and Chanutin[14]	2.71	5.37
Metcoff and Favour[13]	2.77	4.78
Hechter[15]	3.8	6.7
Benditt, Straube, Humphreys	2.6	4.8

concentration of albumin, *i.e.* greater than 1%. Therefore, we have used as a diluent for the dye containing blood samples the albumin solution described above.

There is an inaccuracy of approximately 4% in the hematocrit value as measured by packed cell volume.[9] This error should be relatively constant under constant conditions of packing, and not too large variations in hematocrit. In Table I there is seen a 10% difference between the mean corpuscular hemoglobin concentration of normal and protein depleted rats. From this it is evident that, on the average, a 10% error would be introduced in the erythrocyte volumes of the protein depleted animals if the hematocrits were computed from the hemoglobin as has been suggested.[1] To perform the hematocrit determination in duplicate, as described here, requires only about 0.13 ml of blood. The accuracy attained is adequate. A series of 12 duplicate measurements had a standard deviation of ± 0.85. Nine comparisons of the capillary hematocrits with standard Wintrobe hematocrits yielded an average difference of 0.3%.

In Table I are compared the values of the various blood components for adult male white rats, normal and protein depleted. The data show excellent consistency within each group, as evidenced by the standard errors. In all comparisons the protein depleted animals have values which are significantly lower than those of the controls.

In Table II are the mean blood and plasma volumes per unit surface area of adult white rats determined with methods utilizing T-1824. The values given in this paper agree well with those of Metcoff and Favour.[13] Beckwith and Chanutin's[14] plasma volume values also are in agreement with these, but their total blood volumes are greater. Hechter's[15] blood and plasma volumes both are much higher.

We have discussed above the effect of small quantities of protein on T-1824 color. An increase in apparent blood volumes as large as 15% may result from comparing diluted plasma samples containing small quantities of protein with a standard diluted in saline or water. This may explain the high values reported by Beckwith and Chanutin, and Hechter. They unfortunately do not describe their method of standardization.

Summary. A method is described using the dye T-1824 to measure total blood, plasma and circulating erythrocyte volumes in rats. The method employs a single sample of blood taken 5 minutes after the injection of the dye. Certain precautions necessary to obtain accurate measurements of dye in diluted blood samples are discussed. Data are presented for normal adult rats fed (1) an 18% casein diet, (2) and for adult rats fed a low protein diet for 3 months.

[13] Metcoff, J., and Favour, C. B., *Am. J. Physiol.*, 1944, **142**, 94.

[14] Beckwith, J. R., and Chanutin, A., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 66.

[15] Hechter, D., *Endocrinol.*, 1945, **36**, 77.

[16] Lee, M. O., *Am. J. Physiol.*, 1929, **89**, 24.

15417

Abnormalities of Amphibian Development Following Exposure of Sperm to Colchicine.*

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The familiar effects of colchicine alkaloid upon mitosis point to the possible desirability of exposing male and female sex cells to this substance separately. Results of such experiments have recently been reported, for Drosophila, by Hadorn and Niggli.[1] For the grass frog (*Rana pipiens*) they are reported herewith.

In a preliminary experiment sperm were exposed to a commercially prepared emulsion which contained colchicine and a number of other substances. A majority of eggs fertilized by these sperm developed abnormally. This result raised 2 questions: (1) What substance (or substances) in this preparation was responsible for the abnormalities observed? (2) What was its mode of action?

In order to answer the first question, approximately one-half of each testis of a pithed healthy male frog was macerated in tap water containing 0.04% of chemically pure colchicine in solution. The remaining portions of the 2 testes were macerated in tap water without colchicine. Into each of these suspensions, 3 hours later, about 600 eggs were introduced. These were obtained from 2 females induced to ovulate by the method developed by Rugh.[2]

The general result was as follows. Cleavage occurred in about 97% of eggs fertilized by non-treated sperm as compared to 72% fertilized by treated ones. Of the latter group, a very few (about 8%) developed normally; all the rest abnormally. The types of abnormality were apparently not different from those secured by Keppel and Dawson[3] who exposed whole fertilized eggs to colchicine and described the resulting anomalies in detail. The effects also considerably resemble those which many workers have produced by abnormal heat and pressure, by many different organic and inorganic chemicals, and by various surgical procedures. The literature describing such experiments generally reveals 2 frequently recurring results: (1) a single chemical agent can produce many different kinds of abnormality; (2) different numbers of the various types appear in different experiments even when the same treatment is applied. The results of exposing sperm to colchicine did not differ in these respects. Table I gives the numbers of different effects in a representative sample of 100 individuals chosen at random.

A special study was made of cases of partial cleavage. Usually most of the darkly pigmented portion of the animal half cleaved. In some instances, however, less than half of the animal region was involved, and this in a few cases included part of the gray crescent. Since the noncleaving portion might unfavorably affect the cleaving portion, the latter, in 20 cases, was carefully detached from the former by means of the Spemann hair loop. Twelve of these were neuroectodermal fragments. They had been underlain by no invaginated material prior to removal. They lived 3 to 5 days after removal, undergoing no further differentiation. The other fragments included presumptive mesoderm. They lived 4 to 6 days, undergoing both differentiation and regulation into extremely abnormal, histologically complex masses of tissue. In their developmental behavior, these fragments resembled those which have previously been isolated from the corresponding regions of normal individuals and have been studied

* The main substance of this paper was reported at a meeting of the Purdue Biological Society, March 17, 1946.

1 Hadorn, E., and Niggli, H., *Nature*, 1946, **157**, 162.

2 Rugh, R., *Biol. Bull.*, 1934, **66**, 22.

3 Keppel, D. M., and Dawson, A. B., *Biol. Bull.*, 1939, **76**, 153.

TABLE I.
The Number of Individuals Manifesting Each Type of Abnormality Is Indicated in Parentheses.

Cleavage	Gastrulation	Neurulation	Age in days at death	Condition at death
Cleavage fails to occur (28)	Gastrulation fails to occur (28)	Neurulation fails to occur (28)	?	Radical, nonuniform redistribution of pigment
Cleavage occurs in a restricted region (18)	See discussion in text (18)	See discussion in text (18)	3-6	See discussion in text
Total cleavage occurs (54)	Not all of yolk enclosed (40)	Neurulation fails to occur (28)	4-5	Incompleted gastrulae; yolk often vacuolated
		Neural tubes small and deformed (12)	4-16	Small monstrous embryos with much yolk exposed; yolk often vacuolated
	Blastopore closes after neurulation begins (10)	Neural tubes small; closing delayed at posterior end (10)	6-16	Microcephalic monsters; two with spina bifida
	Gastrulation apparently normal (4)	Neurulation apparently normal (4)	14-42	Apparently normal tadpoles
Total (100)	(100)	(100)		

in vitro by Holtfreter,[4] by Hall,[5] and others.

The foregoing experiment was then twice repeated with the single difference that first distilled water, and then physiological salt solution, were substituted for tap water. There was no material difference in the results obtained.

The similarity of these effects to those observed by Keppel and Dawson suggested that abnormalities might result from direct diffusion of colchicine into the egg substance from the sperm water. To eliminate this possibility, portions of 2 testes were macerated in a very small amount (0.2 ml) of 0.04% colchicine solution. After 3 hours, enough tap water was added to reduce the concentration to $2.6 \times 10^{-4}\%$. The remaining portions of the same testes were macerated in the same amount of tap water. To this, after 3 hours, enough dilute colchicine solution was added to effect the same concentration, $2.6 \times 10^{-4}\%$. Eggs were then added at once to both suspensions. As a result of this procedure, only those eggs fertilized by sperm exposed to 0.04% colchicine developed abnormally. From this it is apparent that the effects were *not* produced by direct diffusion of colchicine into the egg substance from the sperm water.

Solution of the second problem, the nature of the role of colchicine in the production of these abnormalities, requires experimental investigation of a number of previous questions. Normal development is usually regarded as consisting of activities within, and interactions between, a self-perpetuating genetical enzyme system and the milieu in which it exists and acts. This indicates that in the present experiment colchicine probably chemically altered: either one or more reactants in the milieu; or one or more substances influencing these reactants; or both. As a first step in selecting among these possibilities, it seemed desirable to ascertain whether colchicine had acted by irreversibly altering something in the sperm prior to fertilization.

Accordingly the previous procedure was repeated. In this instance, however, after reduction of concentration from 0.04% to $2.6 \times 10^{-4}\%$, sperm were allowed to stand for 3 additional hours at room temperature before use. This was done in order to allow free colchicine to diffuse out of the sperm heads. Subsequent observation surprisingly revealed that eggs fertilized by such sperm developed as normally as eggs fertilized by untreated ones. Consequently, unless it be

[4] Holtfreter, J., *Arch. f. Entw-mech.*, 1931, **124**, 404.

[5] Hall, T. S., *J. Exp. Zool.*, 1942, **89**, 1.

supposed that extra standing rendered altered sperm completely incapable of initiating cleavage, which is for many reasons unlikely, it appears that in these experiments colchicine did *not* act by irreversible alteration of anything in the sperm prior to fertilization.

Summary. Eggs of the grass frog manifest various developmental abnormalities after fertilization with sperm previously exposed for 3 hours to 0.04% colchicine solutions. Experimental evidence is presented to show that this is not due to direct diffusion of colchicine into the egg from the sperm water nor to irreversible alteration of anything in the sperm prior to fertilization.

The author expresses thanks to J. Auer, M.D., Department of Pharmacology, St. Louis University School of Medicine, for his generous contribution of a considerable portion of his private supply of chemically pure colchicine alkaloid powder, a substance recently difficult to secure from the accustomed sources.

15418

The Citric Acid and Aconitase Content of the Prostate.

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The prostatic tissue has many properties of special interest. The human prostatic fluid is remarkable for its high content of citric acid and acid phosphatase, while the dog's prostatic fluid is poor in citric acid.[1-3] The growth of the tissue is greatly dependent on the sex hormones which also take part in the regulation of the oxidative phase of carbohydrate metabolism.[4] The human prostate is unable to utilize citric acid and α-ketoglutaric acid. The dog's prostate utilizes both acids, although not as much as the kidney or the heart. Synthesis of citric acid occurs in both tissues.[5]

The large amounts of citric acid found in human prostatic fluid seem to be produced by the prostate as indicated by the large citric acid content of the tissue. As can be seen in Table I, the citric acid content of human hypertrophic adenoma was about 645 mg per 100 g of fresh tissue, the values fluctuating between 1533 and 201 mg. The citric acid content in prostatic cancer was decreased by a factor of almost 10, to about 74.5 mg. The dog's prostate was found to be quite low in citric acid, about 8 mg per 100 g of tissue, *i.e.* 80 times less than the citric acid content of human prostate.

It seems that one of the causes for the large amount of citric acid in human prostate is the presence of powerful transaminations leading to the formation of oxaloacetic acid. The condensation of this dicarboxylic acid with pyruvic acid would give cisaconitic acid which in the presence of aconitase would be hydrated into citric acid. For the formation of citric acid by this process, the prostatic tissue would have to be rich in transaminase and in aconitase. Indeed there is vigorous transamination in the prostatic tissue.[5] Fig. 1 shows that the human prostate is also rich in aconitase, the dog's prostate containing only about half as much. Aconitase was extracted from the tissues by grinding with sand and 5 volumes of 0.1 *M* phosphate of pH 7.4. The suspension was centrifuged and the clear supernatant fluid was diluted so that it contained around 5 mg of tissue. To 4 cc of this solution was added 4 cc of 0.92 *M* cisaconitic

1 Schersten, B., *Skand. Arch. Physiol.*, 1936, **74**, Supp. 9.

2 Dickens, F., *Biochem. J.*, 1941, **35**, 1011.

3 Huggins, C., and Neal, W., *J. Exp. Med.*, 1942, **76**, 527.

4 Barron, E. S. G., and Huggins, C., *J. Urol.*, 1944, **51**, 630.

5 Barron, E. S. G., and Huggins, C., *J. Urol.*, 1946, **55**, 385.

TABLE I.
Citric Acid Content of Prostatic Tissue.
(Mg per 100 g of tissue).

	Human	Dog
Benign hypertrophy	218	6.3
	589	8.1
	1533	3.3
	523	9.0
	614	12.0
	201	7.0
	720	5.0
	566	10.0
	462	
	1167	
	915	
	1006	
	331	
	426	
	414	
Cancer	105	
	44	
	12	
	137	

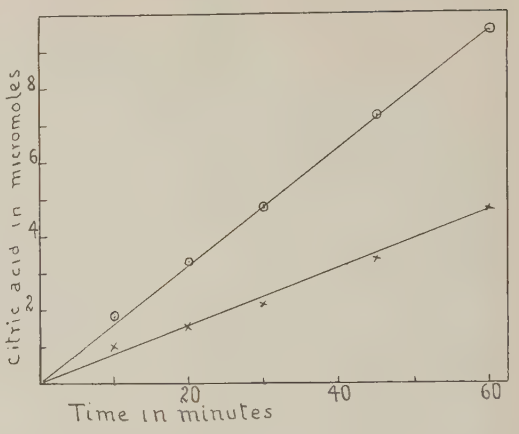

FIG. 1.

Aconitase in prostatic tissue. Enzyme extracted with phosphate buffer of pH 7.4. Cisaconitic acid concentration, 2.3×10^{-2} *M*. Incubation, 37.5°. Abscissa, incubation time in minutes; ordinate, citric acid formation in micromoles. The enzyme solution was that contained in 4 mg of fresh tissue.

acid neutralized in ice-cold water with solid $NaHCO_3$, 4 cc of 0.1 *M* phosphate buffer of pH 7.4 and 4 cc of water. The samples were incubated at 37.5° and the citric acid formed was determined at different intervals for 60 minutes. As the amount of cisaconitic acid was in excess, and the enzyme concentration was low, citric acid formation was linear. In 60 minutes 9.5 micromoles of citric acid were formed by the aconitase from human hypertrophic prostate; aconitase from dog's prostate produced 4.7 micromoles. According to Johnson[6] the aconitase content of tissue extracts, expressed as cmm citric acid formed per mg dry weight per hour (Q aconitase) is as follows: pigeon breast muscle, 120; liver (rat) 61.5; kidney (rat) 80; testis (rat) 7.6; lung, (rat) 14.5; brain, 10. The Q aconitase value of human hypertrophic prostate was 74 and that of dog's prostate, 43. The aconitase content of human prostate is thus almost as high as that of kidney and 10 times as high as that of testis.

The lack of citric acid oxidation, the powerful transamination, and the high aconitase content contribute to the accumulation of citric acid in the human prostatic tissue.

Summary. The citric acid content of human prostatic adenoma was about 645 mg per 100 g of tissue; that of human prostatic cancer, 74 mg; and that of dog's prostate, 8 mg. The human prostate was found rich in aconitase while that of dog's prostate contained half as much of this enzyme. The source of citric acid in prostatic tissue has been postulated.

[6] Johnson, W. A., *Biochem. J.*, 1939, **33** 1046,

15419

Loss of Fertilizing Power of Sea-Urchin and Urechis Sperm Treated with "Univalent" Antibodies vs. Antifertilizin.*

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The substance on sperm that reacts with the natural sperm agglutinin (fertilizin) has been termed[1] antifertilizin. It has been extracted and purified[2] and its role in fertilization examined[3] by a study of the fertilizing capacity and respiration of spermatozoa from which it had been partially extracted. It has also been shown[3] to be an active antigen capable of eliciting antibody formation in rabbits. This suggested the use of rabbit antisera for obtaining further information concerning its role in fertilization. The results of the present investigation show that such antisera, treated so as to render them non-agglutinating, destroy to a great extent the fertilizing capacity of the spermatozoa without appreciably affecting their motility.

Material and Methods. Antifertilizin solutions were prepared from sperm of the sea-urchin *Lytechinus pictus* and the gephyrean worm *Urechis caupo* by extraction with pH 3-4 sea water and purified by dialysis, salting out with saturated ammonium sulfate and isoelectric precipitation in the absence of salts. Examined electrophoretically the preparations showed only one component. The solutions of antifertilizin used for immunization contained 0.2 to 0.3 mg N/ml. These solutions titered to 128 to 256 in the egg-agglutination reaction. The rabbits (4 for Lytechinus and 2 for Urechis) were each given an alternate-day series of 6 to 10 injections with a total of 12 to 22 ml of the solutions. The antisera, obtained 4 to 8 days after the last injection agglutinated homologous sperm, the titers with 1% sperm suspensions ranging from 256 to 2048.

Experimental. Sea-urchin or *Urechis* sperm treated with homologous antiserum is found to be relatively ineffective for fertilization. This might well be expected on the basis simply of a mechanical effect of the agglutinin which ties up the sperm in clumps. To test for specific effects of antibodies on fertilizing capacity of sperm, it is desirable, then, to avoid the agglutinating action. In a preliminary previous test[3] this was done to some extent by the use of dilute sperm with excess antiserum and an inhibiting effect on fertilizing capacity was obtained. More recently, a fairly effective method, photo-oxidation, has been found[4] for converting antibodies into a non-agglutinating, but still specifically reactive form, termed "univalent." This provided an opportunity for further investigation of the problem.

Samples of the antisera, having agglutinative titers of 512 to 2048, were subjected to photo-oxidation as previously[4] described using 0.2% eosin as photo-sensitizer. The amount of photo-oxidation required to destroy agglutinating action in different samples of antisera varied from O_2 uptakes of 200 to 500 mm^3/ml. Since conversion of the antibodies to the univalent form evidently[4] involves a general interchange of parts of the various serum proteins, the variation may

* This work has been aided by a grant from The Rockefeller Foundation. I am indebted to Miss Margaret L. Campbell for technical assistance.

1 Lillie, F. R., *J. Exp. Zoöl.*, 1914, **16**, 523.

2 Frank, J., *Biol. Bull.*, 1939, **76**, 190; Tyler, A., *Proc. Nat. Acad. Sci.*, 1939, **25**, 317; Tyler, A., *West. J. Surg. Obst. and Gyn.*, 1942, **50**, 126; Tyler, A., *Proc. Nat. Acad. Sci.*, 1942, **28**, 391; Hartmann, M., Schartau, O., and Wallenfels, K., *Biol. Zentralblatt*, 1940, **60**, 398; Runnström, J., Tiselius, A., and Lindvall, S., *Ark. f. Zool.* (Stockholm), 1945, **36**A, No. 22, 1.

3 Tyler, A., and O'Melveny, K., *Biol. Bull.*, 1941, **81**, 364.

4 Tyler, A., *J. Immunol.*, 1945, **51**, 157; Tyler, A., *J. Immunol.*, 1945, **51**, 329; Tyler, A., and Swingle, S. M., *J. Immunol.*, 1945, **51**, 339.

TABLE I.

Fertilization of Lytechinus and Urechis Eggs by Homologous Sperm Treated with "Univalent" Antibodies.

Percentage fertilization of *ca.* 400 eggs in 5 ml of sea-water by sperm treated 15 minutes with photo-oxidized rabbit-antisera vs. antifertilizin of Lytechinus (L1 and L2) and of Urechis (U1 and U2) and normal rabbit-serum (N1), using 1 vol. of 1% sperm to 9 vols. of half-strength serum adjusted to sea-water salinity. Photo-oxidation: 325 mm^3 O_2 uptake per ml serum for L1, U1, N1; 410 mm^3 for L2, U2. Original agglutinative titers: 512 for L1 and L2; 1024 for U1; 2048 for U2. Titers for inhibition of agglutination: 6 for L1; 9 for L2; 8 for U1; 12 for U2.

Ml of serum-treated sperm (0.1%) used for insemination	Eggs and sperm of Lytechinus														Eggs and sperm of Urechis					
	Test 1			Test 2			Test 3		Test 4		Test 5		Test 6		Test1		Test 2		Test 3	
	L1	N1	U1	L1	N1	U1	L1	N1	L1	U1	L2	U2	L2	U2	U1	L1	U2	L2	U2	L2
0.013	—	—	—	—	—	—	0	10	0	35	—	—	—	—	½	20	—	—	0	2
0.025	0	0	0	0	10	15	0	15	0	60	0	25	0	50	2	55	0	60	0	10
0.05	0	0	0	0	30	35	0	45	0	95	1	70	½	90	5	95	0	90	0	30
0.1	0	10	4	0	85	75	0	75	0	100	1	75	5	100	12	100	1	90	0	45
0.2	0	25	50	0	95	99	½	85	0	100	2	90	15	99	15	99	0	85	½	80
0.4	0	65	85	0	100	100	1	99	2	100	5	100	15	99	30	100	5	90	½	100
0.8	0	95	99	1	100	99	—	—	—	—	20	100	40	99	—	—	10	90	1	100

be due to differences in their relative amounts and properties in the different samples. After photo-oxidation beyond the point where agglutinating action was lost, the samples of antisera were tested for the presence of "univalent" antibodies by the inhibition method.[4] Several samples were thus obtained that had inhibition titers (amount of treated serum required to prevent the agglutinating action of one volume of untreated, agglutinating, serum) ranging from 4 to 12 and these were employed for treatment of the sperm.

Several things complicate tests of this sort with sperm. It is well known that the fertilizing capacity of sperm decreases with age of the suspension and that the rate of decrease varies inversely with the concentration. Also, it is obvious that the use of too little or too much sperm would obscure possible effects on fertilization and it is important that the range of amounts of sperm used for insemination cover or approximate the minimum at which the control gives 100% fertilization. At the same time it is desirable to treat the sperm with an excess of antibodies. Further, sperm from different animals may vary considerably in fertilizing capacity. Although it was possible to standardize sperm suspensions, eggs and procedure to some extent, only one-fourth of the tests covered the proper range.

In the tests one volume of 1% sperm suspension (1 vol. dry sperm plus 99 vols. sea-water; turbidity checked photoelectrically) was mixed with 9 volumes of serum that had been adjusted to the salinity of sea-water by the addition of an equal volume of concentrated (1.73-fold) sea-water. After 15 minutes various amounts of the sperm-serum mixtures were added to dishes containing aliquot samples of freshly washed eggs (ca. 400 eggs per dish) in a volume of 5 ml. The eggs were examined after ½ hour for membrane elevation and after 2 hours for cleavage.

The results of 6 sets of experiments with Lytechinus and of 3 with Urechis are given in Table I. In these tests the control sera consisted of similarly photo-oxidized heterologous antisera, *i.e.,* U1 and U2 (anti-Urechis) for Lytechinus and L1 and L2 (anti-Lytechinus) for Urechis as well as N1 (normal rabbit-serum). The variability mentioned above, is illustrated in the results of the different tests in which the same serum and sperm concentrations were employed. However, for the present purpose, comparisons need only to be made within each test. On this basis, it may be seen from Table I that the spermatozoa treated with homologous serum give considerably less fertilization than do those treated with control serum. In certain of the tests (Lytechinus tests 5 and 6, Urechis tests 1 and 3) the smallest amounts

of control serum-treated sperm gave percentages of fertilization that approximated those obtained with the largest amounts of homologous antiserum-treated sperm. From these and from the other tests by extrapolation an estimate may be made of the reduction in fertilizing capacity of the sperm due to treatment with antiserum. This reduction ranges from 32-fold in 3 of the tests (Lytechinus 5 and 6, Urechis 1) to considerably more than 64-fold in the others. The difference apparently correlates with differences in antibody-titer (based on both original agglutinative titers and inhibitive titers) but more extensive tests would be necessary for the determination of any quantitative relation. The present results suffice to demonstrate that treatment of sperm with antisera can effect a greater than 64-fold reduction in fertilizing power.

In each of the tests the spermatozoa were examined microscopically for possible effects of antiserum on their motility. No noticeable differences from the activity of the spermatozoa in the control sera were observed, about 70 to 90% of the sperm being highly active in both.

Discussion. Since the antibodies employed in these tests were produced as a result of immunization of rabbits with antifertilizin obtained from the spermatozoa, it would appear that the inhibiting effect on fertilization furnishes further support of the view that the antifertilizin is intimately involved in the fertilization process. It does not, however, necessarily follow that the specific groups or structures of the antifertilizin molecules that may be involved in the initial reaction of sperm and egg are the same as those that serve as the active antigenic determinants in producing antibodies in the rabbit. It is quite conceivable that the antibodies are directed against other specific groups, and their presence on the surface simply blocks the action of those involved in the reaction with fertilizin, etc. Tests that are planned with closely related, difficultly cross-fertilizing, species may help decide this point.

In the present experiments the antibodies were first converted to a non-agglutinating "univalent," form in order to eliminate the mechanical effect of the clumping. It may be noted that such "univalent" antibodies may provide a useful tool in various types of immunological investigations involving enzymes, hormones, bacterial antigens, viruses, etc., where it is desirable to avoid the agglomerating action of the ordinary, "multivalent," antibodies.

Summary. Antisera were produced in rabbits by immunization with purified antifertilizin of Lytechinus and of Urechis and were photo-oxidized so as to convert the antibodies into the "univalent" (non-agglutinating) form. Sperm treated with such antisera were found to possess less than 1/64 of the fertilizing capacity of the controls although their activity was not noticeably affected. The bearing of the results on the significance of antifertilizin in the fertilization reaction is briefly discussed and the possible general use of "univalent" antibodies is mentioned.

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Influence of Amino Acid Feeding upon Antibody Production in Protein Depleted Rats.*

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(Introduced by Paul R. Cannon).

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In previous publications from this laboratory the deleterious effects of prolonged protein depletion upon antibody formation in rabbits and rats have been reported.[1,2] In

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man the association of famine and infectious disease[3] as well as the many examples of increased susceptibility to intercurrent infections evidenced by hypoproteinemic patients with chronic debilitating diseases[4] indicate a relationship between malnutrition and reduced resistance to infection. Furthermore, a correlation between a low gamma globulin level and an inability to form antibodies has been recently demonstrated.[5]

We have attempted to correct the reduced capacity to produce antibodies in protein depleted rats and have found that the feeding of adequate amounts of high quality protein will quickly lead to a restoration of the antibody producing mechanism.[6] Of particular interest was the finding that a casein hydrolysate (Amigen), when fed in adequate amounts, led to a marked improvement in antibody formation relative to that of hypoproteinemic rats fed a similar and isocaloric low protein ration in equivalent amounts. It appears likely, therefore, that restoration of the antibody forming mechanism resulted from the utilization of amino acids in the hydrolysate. This conclusion is strengthened by the following experiment in which crystalline amino acids were fed as the source of dietary amino nitrogen.

Experimental. The methods and rations used in inducing protein depletion in the rat, as well as the techniques of bleeding, serum protein and hemoglobin determinations, and hemolysin titration have been described.[2-6] In this experiment 3 comparable groups consisting each of 6 young adult male rats were

TABLE I.
Summary of Experimental Data.

No. of rats per group	Avg initial wt, (g)	Depletion period			Avg values at start of repletion period			Repletion period			
		Ration	Days	Avg wt loss or gain, (%)	Avg wt, (g)	Avg serum prot., (g%)	Avg Hb., (g%)	Ration	Days before antigen inj.	Gain or loss after 7 days: Avg wt (g)	Gain or loss after 7 days: Avg serum prot., (g%)	Gain or loss after 7 days: Avg Hb., (g%)
6	212	3E	173	—35	137	4.64	10.5	4E	7	— 9	—0.38	—0.6
6	211	3E	173	—34	140	4.56	10.1	AAR	7	+12	+1.75	+3.7
6	212	3C	173	+52	319	7.13	16.2	4C	7	—15	—0.05	+0.2
6	208	3E	173	—35	136	4.52	9.4	AAR + liver	7	+14	+1.68	+3.8

Livestock and Meat Board, the Douglas Smith Foundation of The University of Chicago, and Allen B. Wrisley and Co.

[1] Cannon, P. R., Chase, W. E., and Wissler, R. W., *J. Immunol.*, 1943, **47**, 133.

[2] Cannon, P. R., Wissler, R. W., Woolridge, R. L., and Benditt, E. P., *Ann. Surg.*, 1944, **120**, 514.

[3] Cannon, P. R., *Sci. Monthly*, 1943, **56**, 5.

[4] Cannon, P. R., *J. Am. Diet. Assn.*, 1944, **20**, 133.

[5] Krebs, E. G., *J. Lab. and Clin. Med.*, 1946, **31**, 85.

[6] Wissler, R. W., Woolridge, R. L., Steffee, C. H., Jr., and Cannon, P. R., *J. Immunol.*, 1946, **52**, 267.

fed a low protein ration (diet 3E) for a depletion period of about 6 months, while an additional control group was fed a similar ration adequate in protein (diet 3C). After weighing these animals and determining their concentrations of serum protein and hemoglobin (Table I) one depleted group was placed on a practically protein-free ration (diet 4E; N x 6.25 = 0.88%). A second group received this same ration into which a mixture of 16 crystalline amino acids* modeled after the amino acid composition of casein was incorporated, by replacing an equivalent amount of cornstarch with 36.6 g of amino acids per 100 g of ration (diet AAR; N x 6.25 = 26.5). In addition the liver concentrate (Wilson & Co. 20:1) was omitted and the amounts of niacin, riboflavin, pyridoxine HCl, Ca Pantothenate and Choline Cl were increased in order to equalize the concentrations of these vitamins in the two rations. The third group of depleted rats was fed this second ration with the addition of 1% of liver concentrate (AAR plus Liver; N x 6.25 = 26.8). During this repletion period the control animals were fed a ration (diet 4C) which was similar to the low protein ration except that vitamin test casein was substituted for an equivalent amount of cornstarch (N x 6.25 = 21.4%). The animals were housed in individual wire-bottomed cages and their food intakes were carefully equalized by the paired feeding technique. Their daily diet consumptions averaged approximately 8.5 g per rat per day or about half the amount we have usually fed rats during the repletion period. After 7 days of repletion all the rats were weighed and bled for base line hemolysin titers as well as serum protein and hemoglobin concentrations. The average changes in weights, serum protein and hemoglobin concentration are summarized in Table I where it can be seen that the rats consuming the 4E ration continued to lose weight and concentrations of serum protein and hemoglobin while the rats fed the 4C ration lost weight but maintained a rather constant level of serum protein and hemoglobin. On the other hand the two groups fed the amino acid rations gained a small but significant amount of weight during the 7 days in spite of their restricted food intake. Simultaneously they made marked gains in serum protein and hemoglobin concentrations. There was no significant difference between the response of the rats receiving the amino acid ration without liver (AAR) as compared to the group receiving this ration with liver (Table I).

On the seventh day of repletion each animal was injected intravenously with 1.0 ml of 0.25% suspension of washed sheep erythrocytes. Blood for hemolysin titrations was obtained on the fourth, sixth and tenth days after injection. The average antibody curves are shown in Fig. 1. It is evident that the substitution of pure crystalline amino acids for carbohydrate with the other ingredients kept constant and the diet intakes equalized, caused a definite increase in antibody production, the average titers of the amino acid fed rats being from 4 to 5 times higher than those of the animals maintained on the low protein ration. Furthermore, there was little difference in antibody formation between the group which received liver concentrate and the group which did not. The average hemolysin titers of the control animals receiving diet 4C were considerably higher than those of any of these groups and averaged 340, 4160 and 768 on 4, 6 and 10 days respectively. As might be expected, the animals on the repletion regime had not regained their "normal" antibody producing capacity after only one week of re-feeding with amino acids when this was accompanied by a rather marked overall food restriction. It is probable that these animals were forced

* Grams of amino acids per 100 g of diet:

Arginine, l (+) HCl	1.536
Histidine, l (+) HCl · H_2O	1.046
Lysine, l (+) HCl · H_2O	2.935
Tryptophane, dl	0.558
Phenylalanine, dl	1.611
Methionine, dl	1.084
Threonine, dl	2.417
Leucine, l (—)	3.749
Isoleucine, dl	4.028
Valine, dl	4.338
Tyrosine, l (—)	1.983
Cystine, l (—)	0.112
Glutamic acid, l (—)	7.347
Aspartic acid, dl	1.952
Glycine	0.155
Alanine, dl	1.735
Total g amino acid/100 g diet	36.590

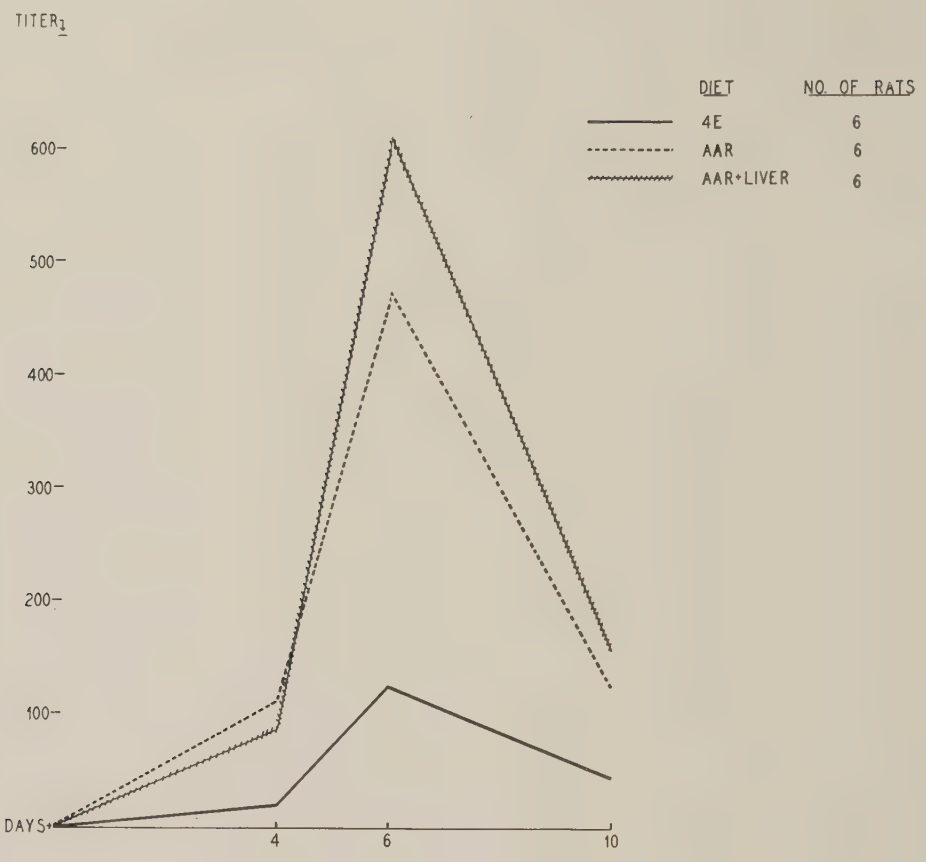

FIG. 1.
Average hemolysin titers at four, six, and ten days.

to utilize much of their ingested amino acids for energy metabolism. This probability is strengthened by the fact that in other experiments in this laboratory (to be published), protein depleted rats fed similar amino acid rations at a level of approximately 15 g per rat per day have consistently shown a weight gain of about 35 g in a 7 day period, even though the amino acid mixture was fed at a level of only 12.1 g per 100 g of diet. Furthermore, in investigating the relationship between caloric intake and the utilization of dietary protein we have found that the level of food consumption employed in this experiment markedly limits the depleted animal's weight gain and blood protein regeneration. Therefore, the results of this experiment probably do not represent the greatest degree of repletion of weight, serum protein, hemoglobin and antibody forming capacity which would be possible when amino acids are fed as the source of dietary amino nitrogen. These results do speak, however, for a recovery of a part of the lost capacity of protein depleted rats to fabricate antibody globulin when the only variable in the diets is the substitution of a mixture of amino acids for carbohydrate.

Others have reported recently that the growth of young animals is retarded when pure amino acids or completely hydrolyzed proteins are fed as the sole source of amino nitrogen, unless a factor (strepogenin) present in native proteins, liver concentrate or partially hydrolyzed protein is supplied.[7,8]

[7] Woolley, D. W., *J. Biol. Chem.*, 1946, **162**, 383.

[8] Womack, M., and Rose, W. C., *J. Biol. Chem.*, 1946, **162**, 735.

There was little evidence under the conditions of our experiment that the presence of factors in liver concentrate other than the vitamins mentioned above exert any appreciable effect upon the repletion of the depleted rat's tissue and blood proteins.

These results strengthen our view that antibody production depends upon an adequate supply of essential amino acids coming either from food or the animal's own protein stores. When both of these sources are markedly diminished the antibody producing capacity is markedly reduced. This capacity can be re-established by feeding adequate and well balanced quantities of amino acids coming from food protein, enzymatic protein hydrolysates, or amino acid mixtures.

15421

Increased Serum Phosphatase and "Hyperprothrombinemia" in Infectious Hepatitis of Children.

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The alkaline phosphatase activity of serum and the plasma prothrombin time are among the most commonly used indices of hepatic function. The phosphatase finds its most common application in the differential diagnosis of jaundice, where some observers believe the level of enzyme activity to be one of the most useful criteria in the differentiation of obstructive from hepatogenous jaundice.[1-3] Others,[4-8] while confirming that marked increases of phosphatase activity are more frequently found in obstructive than in "catarrhal" disorders of the liver, have observed overlapping to a considerable extent between the 2 groups. As to the behaviour of serum phosphatase in infectious hepatitis, results and conclusions of different observers are somewhat at variance. That some elevation of phosphatase occurs, appears evident from the figures of most studies.[1-9] While according to some, the increase of enzyme activity is only slight,[1-3] others[4-7,9] have frequently found greater rises. It has been suggested[1,2,5] that children may react during infectious hepatitis with higher phosphatase levels than adults, a suggestion in keeping with the results of one study, the subjects of which were for the most part children.[9]

In the study of the plasma prothrombin time, the greatest attention has been paid to the occurrence of hypoprothrombinemia. Lowering of the plasma prothrombin in patients with hepatic disease may be caused by a deficiency of vitamin K owing to decreased intestinal absorption or by impairment of the ability of the liver to form prothrombin. Two practical applications of the prothrombin time have been in the foreground: 1. as an index of bleeding tendency, and 2. as a

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[2a] Flood, C. A., Gutman, E. B., and Gutman, A. B., *Arch. Int. Med.*, 1937, **59**, 981; [2b] Gutman, A. B., Olson, K. B., Gutman, E. B., and Flood, C. A., *J. Clin. Invest.*, 1940, **19**, 129; [2c] Gutman, A. B., and Hanger, F. M., Jr., *M. Clin. North America*, 1941, **25**, 837.

[3] Rothman, M. M., Meranze, D. R., and Meranze, T., *Am. J. Med. Sc.*, 1936, **192**, 526.

[4] Herbert, F. K., *Brit. J. Exp. Path.*, 1935, **16**, 365.

[5] Shay, H., and Fieman, P., *Am. J. Digest. Dis.*, 1938, **5**, 597.

[6] Giordano, A. S., Wilhelm, A., and Prestrud, M. C., *Am. J. Clin. Path.*, 1939, **9**, 226.

[7] Greene, C. H., Shattuck, H. F., and Kaplowitz, L., *J. Clin. Invest.*, 1934, **13**, 1079.

[8] Cantarow, A., and Nelson, J., *Arch. Int. Med.*, 1937, **59**, 1045.

[9] Bodansky, A., and Jaffe, H. L., PROC. SOC. EXP. BIOL. AND MED., 1933, **31**, 107.

TABLE I.
Indices of Hepatic Function in Infectious Hepatitis of Children.

Case No.	Age, yrs	Symptoms before onset of jaundice	Serum: Approximate time since onset of jaundice, days	Serum: Bilirubin., mg per 100 cc	Serum: Cephalin flocculation	Serum: Thymol turbidity, units	Serum: Phosphatase, Bodansky units per 100 cc	Plasma: Prothrombin time undil. seconds	Plasma: Prothrombin time dil. const. seconds
1	9	6 days prior, vomiting	1	3.8	+++	8		16.5	4.1
			2		+++	12		21.8	3.6
			6	1.2	+++	12	15	33.6	6.3
			12	0.7	++	5	5	19.6	3.7
2	11	5 days prior, abdominal pain	1	3.8	+++	13		20.9	3.6
			4	2.1	+++	16	16	23.5	5.4
			8	1.0	++++	20	8	22.2	4.4
			16	0.2	+++	10	5		
3	10	8 days prior, epigastric pain and vomiting	3	6.9	+++	22	14	18.8	2.4
			7	2.0	+++	20	12	25.2	3.1
			15	1.0	++++	12	5	27.0	3.9
4	14	10 days prior, anorexia and fever	1	4.8	+++	20	17	17.8	3.6
			3	5.2				18.6	3.6
			6	3.8	+++	20	15	25.2	5.2
			26	0.3	++		6	20.2	3.6
5	13	2 days before examination, fever. Bilirubinuria on admission	0	1.0	+++	20			
			3	0.7	+++	15	19		
			5	0.7	++++		12	23.3	4.8
			8	0.6			10	27.3	5.2
6	10	7 days prior, fever	1	8.5	+++			14.7	2.7
			4	4.0			37	16.8	2.4
			7	2.3	+++			18.7	3.3
			10	1.8			8	25.3	3.8
7	9	6 days prior, fever, nausea	1	4.9	+++		26	16.4	1.3
			21	0.5	++		8	19.8	4.0
8	9	6 days prior, headache and abdominal pain	1	2.7	+++		17	16.4	2.1
			26	0.5	++		6		
9	10	None	8	3.0	++++		15	17.1	
			23	0.5	++		7		

10	11	4 days prior, epigastric discomfort	2	11.8	++	18	16.0	0.5	
			10	2.7		8			
11	7	5 days prior, epigastric pain and vomiting	1	4.8	++		16.8	2.9	
			2				17.0	2.5	
12	14	8 days prior, fatigue and nausea	5	7.8	+++	25	20.6	0.8	
			15	0.7	+++	7			
13	11	8 days prior, epigastric pain; 3 days prior, epistaxis	1	4.4	++		16.1	2.4	

measure of hepatic function, when vitamin K is supplied in adequate amounts. In infectious hepatitis, moderate degrees of hypoprothrombinemia have been found. A common observation is that, when the disease is severe, the usually present hypoprothrombinemia does not respond readily to vitamin therapy.

In this communication are presented observations on 13 children on whom determinations of phosphatase and prothrombin time, together with other tests of liver function, were obtained in the early stages of infectious hepatitis. In these cases transient elevations of considerable magnitude in the activity of phosphatase were observed. In the majority of instances abnormally short prothrombin times were noted during the first days of illness. These soon gave way to prolonged prothrombin times. The findings, aside from their limited practical value, are of interest in the consideration of the mechanism and the pattern of change of hepatic function in disorders of the liver.

Material and Methods. The patients studied were 13 children, 7 of whom were girls, varying in age from 7 to 14 years. Before the onset of hepatitis they had been well, and they gave no history of relevant antecedent disease. Their symptoms were mild in nature, and complaints were elicited in some only on questioning at routine visits to the outpatient department. Generally, 2 to 8 days before the onset of jaundice untoward manifestations, such as fever, anorexia, abdominal pain, or vomiting had occurred. Eight of the children were examined the day after the jaundice had been noticed, others 2, 3, 5 and 8 days afterwards. One patient, who may be classified as having exhibited hepatitis without jaundice, never presented clinical icterus. Bilirubinuria was found in all instances. The stools of 2 of the patients appeared lighter in color than normal.

Serum bilirubin was determined according to the method of Malloy and Evelyn,[10] alka-

[10] Malloy, H. T., and Evelyn, K. A., *J. Biol. Chem.*, 1937, **119**, 481.

[11] Bodansky, A., *J. Biol. Chem.*, 1933, **101**, 93.

[12] Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, **66**, 375.

line serum phosphatase according to the procedure of Bodansky,[11] except that the method of Fiske and Subbarow,[12] modified for the photoelectric colorimeter, was used to measure the inorganic phosphate. Prothrombin time was determined by a slightly modified method of Fullerton,[13] with Russel viper venom as thromboplastin. The prothrombin time of undiluted plasma as well as of several plasma dilutions was tested. The data of the dilution curves for each plasma were expressed by 2 numbers: 1. the clotting time of undiluted plasma, and 2. the dilution constant expressing the increase in seconds per unit increment of reciprocal dilution. The use of a constant is based on the finding of a linear relationship between the clotting time and the reciprocal of the dilution for any given plasma (unpublished data). The mean normal value, based on the data of 40 subjects, for the prothrombin time of undiluted plasma was 19.8 sec. $\pm$ a standard deviation of 1.2 sec. and for the dilution constant 3.8 $\pm$ 0.8 sec. Accepting the customary limits of $\pm 2 \times$ S.D. as the normal range, values below 17.5 and over 22.3 seconds for the undiluted plasma clotting time and values below 2.1 and over 5.5 for the dilution constant may be considered abnormal. The cephalin flocculation test was carried out and read according to Hanger,[14] with precautions to avoid exposure to light. Only the 24-hour readings were recorded. The thymol turbidity test was performed according to Maclagan.[15] Values over 4 units according to the author of the method are considered to be abnormal.

Results and Discussion. The high levels of phosphatase activity observed in the patients here presented are comparable to those reported on a group of patients of similar age distribution.[9] That some increase of enzyme activity, although perhaps of smaller degree, occurs in adult patients, is evident from the data of other studies.[1-8] The transient nature of the increase is apparent from the data on cases 1 to 3, and 5, in which normal values were found when the determinations were repeated 2 to 6 days after a high phosphatase value had been determined. The evanescent nature of the rise of phosphatase activity, although not stressed, is also indicated from an inspection of the data of previous reports.[2,9] The practical importance of these observations is limited, but they indicate that any interpretation of single determinations of phosphatase activity should be made cautiously.

The degree and pattern of the increase in phosphatase aside from its diagnostic aspect, is of interest in the consideration of the mechanism of this phenomenon. According to the "retention" theory,[2] most of the serum phosphatase activity is osteogenous. This view is based on the finding of high enzyme activity in bone tissue, and on changes observed in the serum enzyme activity in experimental conditions and disorders affecting the skeletal metabolism. The primary function of the liver, according to this theory, is excretion of the serum phosphatase, an assumption based on the high enzyme activity of bile. The elevation of phosphatase in obstructive jaundice is ascribed to interference with the enzyme excretion. By the same token, an elevated phosphatase level is taken as a sign of impairment of the excretory function of the liver. Another explanation[9] for the behaviour of the serum phosphatase is that at least part of the normal activity is hepatogenous, and that the rise in the enzyme activity in hepatic disease is caused by stimulation of the liver cell. The variable extent of the increase is explicable on the assumption that the formation of the phosphatase is variably influenced by different types of noxious influence on the liver cell. The data here reported appear to favor the view that the elevation of the enzyme activity represents a characteristic response of the liver to a certain type of damage. Its occurrence in the early phase of the disease, as well as the dissociation between bilirubin level and extent of increase of the enzyme activity, suggest that the rise in phosphatase reflects a stimulation rather than a suppression of the activity of the liver cell. The incidence of considerably increased

[13] Fullerton, L., *Lancet*, 1940, **2**, 195.

[14] Hanger, F. M., *J. Clin. Invest.*, 1939, **18**, 261.

[15] Maclagan, N. F., *Brit. J. Exp. Path.*, 1944, **25**, 234.

phosphatase activity, more frequent in obstructive than in hepatogenous jaundice, may be explained at least in part by the assumption that biliary obstruction in most instances represents a peculiarly effective stimulus to the liver cell. The finding of normal or only moderately increased phosphatase levels in infants with congenital atresia of the bile ducts[2a,2b,6,8] (unpublished data), indicates that the element of retention, at least in certain types of obstructive jaundice, may play only a minor role. In such patients, on the basis of the "retention" theory one would expect high phosphatase levels, since in this age period the serum phosphatase activity normally is higher than in adult life.

In 8 of the 13 patients abnormally short prothrombin times in undiluted plasma were observed in the first determination. The dilution constant was similarly reduced in most instances to the lower limits of normal, or below. Subsequent determinations, performed within short periods of time in cases 1 to 6, showed variable prolongation of the prothrombin time beyond the upper limits of normal. The interpretation of the abnormally short prothrombin times is as yet uncertain. Some observers have found reduced prothrombin times in animals following large doses of vitamin K,[16] and after the administration of methylated purines,[17] and they have interpreted their findings as an indication of the occurrence of hyperprothrombinemia. This view has been challenged, as such effects have not been noted when the original method of Quick was used.[18] At present it is still undetermined whether the abnormally short prothrombin times reflect an actual increase in prothrombin, or changes in the rate of its activation. An increase in the amount of activators might be involved, or a reduction of the normally occurring clotting inhibitors. If the present view that vitamin K is specifically concerned in the formation of prothrombin is correct, the transient occurrence of short clotting times after large doses of the vitamin,[16] and after therapeutic doses in patients with hypoprothrombinemia (see [16] for references, also unpublished data), would suggest that these changes involve prothrombin *per se*. Whatever the true explanation, the finding of transient "hyperprothrombinemia" indicates that the liver cell reacts to the virus of infectious hepatitis at first with stimulation, followed by depression according to a pattern characteristic for many biologic phenomena. The period of stimulation may be exceedingly short: for instance the prothrombin time in case 1 changed within one day from a low value to one at the upper limits of normal, and increased further to a greatly prolonged value within a few more days. An even more rapid transition was observed in a case of erythroblastosis fetalis (unpublished), where the prothrombin time increased within 12 hours from 8 to 25 seconds.

Summary. Changes of the alkaline phosphatase activity in serum and of plasma prothrombin time were studied in 13 children with infectious hepatitis. Considerable increases of phosphatase and abnormally short prothrombin times were found transiently during the early part of the disease. The "hyperprothrombinemia" soon was followed by hypoprothrombinemia. The findings are interpreted as an indication of stimulation of the liver cell during the first stages of infectious hepatitis.

16 Field, J. B., and Link, K. P., *J. Biol. Chem.*, 1944, **156**, 739.

17 Field, J. B., Larsen, E. G., Spero, L., and Link, K. P., *J. Biol. Chem.*, 1944, **156**, 725.

18 Quick, A. J., *J. Biol. Chem.*, 1945, **161**, 33.

15422 P

Inhibitory Action of Human Epidermis on Melanin Formation.

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Geneticists discovered an inhibitory factor of pigmentation in crude aqueous extracts of guinea pigs' skin.[1] In this laboratory it was found that such a factor also occurs in human epidermis, inhibiting melanin formation in the tyrosine-tryosinase system and preventing the oxidation of *l*-dihydroxyphenylalanin ("dopa") in the air.

Method. Large strips of skin were removed from the abdominal region of white adult cadavers 24 to 48 hours after death. In a few instances fresh skin from female breasts or from amputated limbs were worked up immediately after surgery, but there was no substantial difference between results obtained with fresh and with cadaver skin.

The epidermis was removed according to the method of Baumberger *et al.*[2] by placing the skin on a slide warming table at 56°C with the corium side down. After 3 minutes of warming the epidermis could be easily pulled off. The thin small pieces of epidermis were weighed and sufficient water was added to make a suspension of 100 mg of epidermis per 1.0 cc of water. The suspension was shaken a few times and then placed in the icebox in a stoppered bottle for 24 hours. Next day a turbid or opalescent liquid was decanted and centrifuged. By subsequent ultracentrifugation most of the turbidity could be eliminated without decreasing the activity of the extract.

The extract was added in decreasing concentrations to measured amounts of solutions of tyrosine, tyrosinase and buffer. The pH was 7.4. The test tubes were covered with a cloth and left at room temperature. Results were read in a "Lumetron" colorimeter with blue (420) filter after 24 and 48 hours. The colorimeter was calibrated for measuring melanin in μg per 100 cc aqueous suspension.

Results. Table I shows the inhibition of melanin formation, increasing with the amount of epidermal extract added. Similarly prepared extracts of the corium were ineffective. Table II demonstrates that iodoacetamide, a specific poison for sulfhydryl groups, interferes with the inhibitory action of epidermal extracts. Iodoacetamide alone in similar concentration did not modify melanin formation in the tyrosine-tyrosinase system.

Discussion. Quantitative duplication of these experiments has not been possible, apparently because the epidermis loses varying amounts of water during its separation and weighing. The presence of sulfhydryl compounds in epidermal cells was demonstrated many years ago by histochemical application of the nitroprusside color reaction.[3] Also, it has been known that these compounds inhibit melanin formation.[4] The interference with the inhibitory action of epidermal extracts by iodoacetamide indicates that the inhibition is due to the presence of water-extracta-

TABLE I.

Inhibitory Effect of Epidermal Extract on Melanin Formation.

Each tube contains 9.9 cc of 0.05% tyrosine solution, 0.1 cc of tyrosinase solution (1000 Adams-Nelson units/cc),* 1.0 cc pH 7.4 phosphate buffer, and water sufficient to make a total volume of 12.0 cc.

Tube No.	Epidermal extract, 100 mg/cc cc	Melanin, μg/10 cc after 48 hrs
1	none	178
2	0.05	153
3	0.1	75
4	0.2	40

*Courtesy of the Upjohn Co.

1 Ginsburg, B., *Genetics*, 1944, **29**, 176.

2 Baumberger, J. P., Suntzeff, V., and Cowdry, E. W., *J. Nat. Cancer Inst.*, 1942, **2**, 413.

3 Giroud, A., and Bulliard, H., *La keratinisation de l'epiderme et des phaneres*, G. Doin, Paris, 1930.

4 Figge, F. H. J., and Allen, E., *Endocrinology*, 1941, **29**, 262; Rothman, S., *J. Investigative Dermatology*, 1942, **5**, 67.

5 Anson, M. L., *J. Gen. Physiol.*, 1941, **24**, 399.

TABLE II.
Interference of Iodoacetamide with Inhibitory Effect of Epidermal Extract. Tyrosine, tyrosinase, and buffer same as in Table I. Total volume 13 cc.

Tube No.	Epidermal extract 100 mg/cc cc	Iodoacetamide 24 mg/cc cc	Melanin μg after 24 hrs	48 hrs
1	1.0	0	100	158
2	1.0	1	240	460
3	0.5	0	138	230
4	0.5	1	270	460

ble sulfhydryl compounds in the epidermis. In preliminary experiments the —SH content of our epidermal extracts measured by the ferricyanide method[5] was found to be in the range of 0.0004 millimol of —SH per cc. The —SH content could be substantially depressed by ultraviolet irradiation of excised pieces of skin.

These findings lead to the hypothesis that in melanoblasts both substrate and active enzyme are present but no reaction takes place between them because of the inhibitory action of sulfhydryl compounds. Pigmentogenic stimuli such as sunshine, X-rays, heat and inflammatory cutaneous diseases of unknown origin act by oxidizing or otherwise destroying these compounds, whereupon the enzyme freely acts on the substrate. In addition to postinflammatory pigmentations, hyperpigmentations of endocrine origin may have the same mechanism as suggested by *in vitro* observations of Figge and Allen.[4]

Summary. Aqueous extracts of human epidermis contain substances inhibiting melanin formation in the tyrosine-tyrosinase system. Iodoacetamide interferes with this inhibition. A new hypothesis of the mechanism of postinflammatory pigmentation is presented.

15423

Significance of Histamine in Trypsin Shock as Determined by Benadryl.*

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The following observations have been interpreted as supporting the hypothesis that trypsin owes its toxicity to the action of histamine which is liberated from tissues by this enzyme. Trypsin, like histamine, causes contraction of isolated segments of ileum and uterus from a number of animals.[1-4] This action, like that of histamine, is not inhibited by atropine but is abolished by arginine.[3-6]

Trypsin, like substances known to liberate histamine, when added to rabbit's blood results in a shift of histamine from cells into plasma.[7,8] Rabbits injected intravenously with trypsin show a leucopenia, a fall in total blood histamine, a fall in carotid and a rise in pulmonary arterial pressures,[3,5,9,10]

* This investigation has been made with the assistance of a grant from the Clara A. Abbott Fund of Northwestern University. We are indebted to Parke, Davis and Co. for the benadryl (β-dimethylaminoethyl benzhydryl ether HCl) used in our experiments.

1 Rocha e Silva, M., *Compt. rend. Soc. Biol.*, 1939, **130**, 181.

2 Rocha e Silva, M., *Compt. rend. Soc. Biol.*, 1939, **130**, 184.

3 Rocha e Silva, M., *Arquiv. Inst. Biol. São Paulo*, 1939, **10**, 93.

4 Rocha e Silva, M., *Arch. f. exp. Path. u. Pharm.*, 1940, **194**, 335.

5 Dragstedt, C. A., and Rocha e Silva, M., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 420.

TABLE I.
The Influence of Benadryl on the Toxicity of Trypsin.

Dose trypsin "Difco" (mg/kg)	Control			Treated (10 mg/kg benadryl)		
	No. lived	No. died	% mortality	No. lived	No. died	% mortality
			Guinea Pig.			
80	3	5	63	3	2	40
160	0	4	100	0	4	100
			Dog.			
100	2	7	78	2	7	78

phenomena which are characteristic of the liberation and action of histamine in this species.[11] Trypsin, like histamine and some substances known to release histamine, when injected into the skin of rabbits, gives rise to a positive "trypan-blue reaction."[7,12]

Perfusion of trypsin solutions through the isolated liver of the dog results in the appearance of histamine in the perfusate[13] and the blood of some dogs undergoing trypsin shock shows an elevation in its histamine content.[14]

Perfusion of trypsin solutions through the isolated lung of the guinea pig results in the appearance of histamine in the perfusate.[4,14,15]

There are, therefore, several observations that are consistent with the hypothesis that trypsin owes its toxicity to the liberation and action of histamine. However, there are other observations which are inconsistent with this hypothesis. For example, guinea pigs dying from the intravenous injection of trypsin fail to show either the rise in blood histamine or the emphysematous lungs characteristic of the liberation and action of histamine in this species.[10,16]

The purpose of the present study was to contribute, by the use of benadryl, a potent antagonist to either administered or liberated histamine,[17-20] additional observations bearing on the role played by histamine in the toxicity of trypsin, and to test the adequacy of the existing hypothesis in interpreting these findings.

The trypsin employed in the present studies was a non-crystalline, but potent preparation, sold under the name of "Difco" which has been demonstrated to be free of histamine and to lose all biological activity when treated with trypsin inhibitor from blood plasma. Unless specified it was placed in solution in a borate buffer at pH 7.4.

The first experiments were designed to determine the influence of benadryl on the lethal action of intravenously injected trypsin in the guinea pig and the dog.

Trypsin injected via the jugular vein in a dose of 80 mg per kilo into 8 very lightly anesthetized guinea pigs resulted in the death of 5 or 63% of the animals. Twenty minutes

6 Rocha e Silva, M., *J. Allergy*, 1944, **15**, 399.

7 Rocha e Silva, M., and Dragstedt, C. A., *J. Pharm. and Exp. Therap.*, 1941, **72**, 36.

8 Dragstedt, C. A., Wells, J. A., and Rocha e Silva, M., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 191.

9 Rocha e Silva, M., *Arch. f. exp. Path. u. Pharm.*, 1940, **194**, 351.

10 Rocha e Silva, M., and Essex, H. E., *Am. J. Physiol.*, 1942, **135**, 372.

11 Dragstedt, C. A., *Physiol. Rev.*, 1941, **21**, 563.

12 Rocha e Silva, M., and Dragstedt, C. A., *J. Pharm. and Exp. Therap.*, 1941, **73**, 405.

13 Rocha e Silva, M., and Graña, A., *Arch. Surg.*, in press.

14 Ramirez de Arellano, M., Lawton, A. H., and Dragstedt, C. A., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 360.

15 Rocha e Silva, M., *Compt. rend. Soc. Biol.*, 1939, **130**, 186.

16 Wells, J. A., Dragstedt, C. A., Cooper, J. A., and Morris, H. C., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 57.

17 Loew, E. R., Kaiser, M. E., and Moore, V., *J. Pharm. and Exp. Therap.*, 1945, **83**, 120.

18 Loew, E. R., and Kaiser, M. E., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 235.

19 Wells, J. A., Morris, H. C., Bull, H. B., and Dragstedt, C. A., *J. Pharm. and Exp. Therap.*, 1945, **85**, 122.

20 Wells, J. A., Morris, H. C., and Dragstedt, C. A., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 104.

TABLE II.
Comparative Susceptibility of Blood Pressure Depressing Actions of Trypsin and Histamine in the Dog to 5.0 mg/kg of Benadryl.

	Trypsin "Difco"			Histamine Acid Phosphate		
		Fall in blood pressure (mm Hg)			Fall in blood pressure (mm Hg)	
Dog No.	Dose, mg/kg	Before Benadryl	After Benadryl	Dose, mg/kg	Before Benadryl	After Benadryl
1	.05	34	46	.002	53	1
2	.05	36	36	.001	40	1
3	.25	58	52	.004	54	22
4	1.00	94	78	.100	80	62
5	.05	64	50	.004	60	22
6	.10	70	66	.004	60	6
7	1.00	39	48	.002	40	18
8	.60	54	58	.004	52	20
Avg		59	54		55	19
% decrease			—8			—65

after the intraperitoneal injection of 10 mg per kilo of benadryl, the same dose of trypsin was injected into another group of 5 guinea pigs with the death of 2 or 40%. In similar fashion 2 groups of 4 guinea pigs were injected intravenously with 160 mg per kilo of trypsin with 100% mortality in both the treated and control groups. (Table I). Autopsy of the animals dying from the injection of trypsin confirms previous observations as to the absence of pulmonary emphysema.

Trypsin injected via the femoral vein in a dose of 100 mg per kilo into 9 pentobarbital anesthetized dogs resulted in a precipitous fall in blood pressure to shock levels with death of 7 or 78%. Following the very slow intravenous injection of 10 mg per kilo of benadryl, the same dose of trypsin was injected into another group of 9 dogs with the death of 7 or 78%.

It may be concluded from these experiments that benadryl affords neither the guinea pig nor the dog any significant protection against the lethal action of intravenously injected trypsin.

These findings are definitely inconsistent with the hypothesis that the action of histamine is the sole factor in the toxicity of trypsin. However, they do not deny the possibility that histamine may be one of multiple non-additive factors in the toxicity of trypsin. On the other hand, the liberation of significant amounts of histamine unaccompanied by any evidence of its action is untenable. Therefore, the next experiments were designed to determine the influence of benadryl on the transient fall in blood pressure produced by sublethal doses of trypsin.

A series of 8 pentobarbital anesthetized dogs, arranged for recording blood pressures, were injected with doses of 0.05 to 1.00 mg/kg of trypsin. These doses caused the blood pressure to fall 36 to 94 mm of mercury with complete recovery within 10 minutes. In each instance the fall in blood pressure produced by trypsin was duplicated by a suitable small dose of histamine phosphate. Five mg/kg of benadryl was then slowly injected intravenously and the original doses of trypsin and histamine were repeated.

A comparison of the influence of benadryl on the blood pressure depression produced by these agents reveals (Table II) that in each instance the reduction in the action of histamine is greater than the reduction in the action of trypsin. In 7 of the 8 animals this difference is striking. The average decrease in the blood pressure response to histamine produced by benadryl is 36 mm or 65% and at the same time the decrease in the response to trypsin is only 5 mm or 8%. It should be stated that a moderate amount of tolerance frequently develops to the repeated injection of trypsin and that such an effect would be included in the apparent decrease in the trypsin response as produced by benadryl.

It may be concluded that benadryl is much less effective in reducing the blood pressure

TABLE III.
Blood Histamine in Trypsin Shock in the Dog.

Dog No.	Treatment	Blood histamine (μg histamine base/cc) Before shock	During shock	Outcome
1	None	.12	.07	Died
2	Benadryl (10 mg/kg)	.05	.15	"
3	None	.13	.08	"
4	Benadryl (10 mg/kg)	.06	.06	Lived

response to trypsin than it is in reducing the response to histamine. These observations are also definitely inconsistent with the hypothesis that the action of histamine is the sole factor in the effects produced by trypsin. They cannot be used to deny any participation of histamine in this effect but can relegate it to a minor role, and thus are in direct contrast to those experiments previously reported which claim a substantial increase in blood histamine during trypsin shock in the dog.

Therefore, an experiment was designed to determine the blood histamine before and during trypsin shock in the dog. Blood was drawn from 4 dogs prior to the intravenous injection of 100 mg/kg of trypsin, a dose large enough to cause death in 3 of them. At the time the blood pressure was falling precipitously as a result of the injection of trypsin another sample of blood was drawn from these animals. The trichloracetic acid filtrate of these bloods was subjected to electrodialysis and concentration and assayed for their histamine content on the blood pressure of the atropinized cat. The histamine content of these bloods was expressed (Table III) in terms of mcg of histamine base per cc. It is concluded that in none of these animals was the blood histamine of sufficient magnitude to explain the severity of the reaction produced.

The present studies, therefore, contribute additional information bearing on the role played by histamine in the toxicity of trypsin and these observations are inconsistent with the hypothesis that trypsin owes its toxicity solely to the liberation and action of histamine. It therefore becomes necessary to reconsider the initial observations which have been interpreted as supporting this hypothesis from the point of view that perhaps other interpretations may be made of some of these facts.

The significance of the observation that arginine, a known histamine antagonist, can abolish the action of trypsin on the isolated guinea pig ileum is dependent upon the specificity of arginine as an antagonist. It has been observed that trypsin can liberate a "slow-reacting muscle stimulant substance" which contributes to the response of smooth muscle to trypsin.[21] The fact that arginine completely prevents the action of trypsin on isolated smooth muscle suggests that the "slow-reacting muscle stimulant substance" may also be antagonized by arginine and does not deny the possibility that trypsin itself may be antagonized by arginine. In any event, the specificity of arginine as a histamine antagonist may be questioned and thus the antagonism of the action of trypsin on smooth muscle by this substance need not necessarily be interpreted as indicating histamine to be the mediator of this response.

The specificity of the positive "trypan-blue reaction" for histamine has never been claimed and, in fact, it is known that substances, such as arginine and Kallikrein, which do not cause the liberation of histamine, give a positive test.[22,23] Therefore, a positive "trypan-blue reaction" produced by trypsin need not be interpreted as indicating the liberation and action of histamine.

The previously reported experiments demonstrating a rise in blood histamine in the dog during trypsin shock are inconclusive

[21] Trethewie, E. R., *Australian J. Exp. Biol. and Med. Sci.*, 1942, **20**, 49.

[22] Rocha e Silva, M., and Dragstedt, C. A., *J. Pharm. and Exp. Therap.*, 1941, **73**, 405.

[23] Rocha e Silva, M., *Nature*, 1940, **145**, 591.

in that a significant rise was observed in but one dog, and in view of the present observations, these results must be challenged.

The amounts of histamine obtained from the dog's liver by perfusion of this structure with trypsin solutions are very small, being of the order of 5 to 12 mcg in 30 to 45 minutes of perfusion.

The observed liberation of histamine from isolated guinea pig lungs by perfusion of these structures with trypsin solutions appears to be of no consequence to the toxicity of trypsin in the intact animal in view of the absence of any of the pulmonary effects of histamine when trypsin is injected into the intact animal.

There remains only the observations on the action of trypsin on the blood of rabbits and the action on the intact rabbit which clearly support the histamine hypothesis.

Thus, it is concluded that most of the observations which have been interpreted as supporting the hypothesis that trypsin is toxic because of the action of histamine which it liberates from tissues are susceptible of other interpretation. In addition, such an hypothesis is quite inconsistent with the present observations which indicate that histamine plays no significant role in the toxicity of trypsin.

Summary. It was found that benadryl, a potent antagonist to either injected or liberated histamine, does not modify the lethal action of trypsin in the guinea pig or the dog. It is thus apparent that the histamine hypothesis, relative to the toxicity of trypsin, requires reconsideration.

15424

Studies on the Adherence of the Epidermis to the Corium.

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In some cases of pathologic blister formation, such as epidermolysis bullosa and pemphigus, the separation of the epidermis from the corium cannot be explained by development of pressure due to accumulation of fluid between these layers. In these cases a change in the adhesive forces holding the 2 heterogeneous layers together must be assumed. Histologic studies with modern methods have failed to demonstrate the existence of a basement membrane at the junction of the epidermis and the corium.[1-3] Lately it has been suggested that a decrease in adhesion between the 2 layers is due to physicochemical changes in the upper corium.[4]

It has been known that treatment of the skin with acids or bases results in separation of the epidermis.[5] These types of separation are accompanied by swelling of the corium. In 1942 Baumberger *et al.* discovered that if excised human skin was warmed up to 50°C for a few minutes on a slide warming table, the epidermis could easily be peeled off.[4] When the skin was cooled, the epidermis became reattached to the corium an indication of a reversible gel-sol formation.

Experimental. With regard to the swelling effect of acids, bases, and neutral salts on collagen[6] and the consequent decrease of its

[1] Pautrier, L. M., and Woringer, Fr., *Ann. de Dermatol. et Syph.*, 1930, **7** serie **1**, 985.

[2] Szodoray, L., *Arch. Derm. and Syph.*, 1931, **23**, 920.

[3] McLeod, J. M. H., and Muende, I., *Pathology of the Skin*, 1940, New York, N. Y., Paul B. Hoeber, Inc.

[4] Baumberger, J. P., Suntzeff, V., and Cowdry, E. V., *J. Nat. Cancer Inst.*, 1942, **2**, 413.

[5] Wilson, J. A., *The Chemistry of Leather Manufacture*, 1928, New York, N. Y., The Chemical Catalogue Co.

[6] Lloyd, D. J., and Shore, A., *Chemistry of the Proteins*, 1938, London, J. and A. Churchill Ltd.

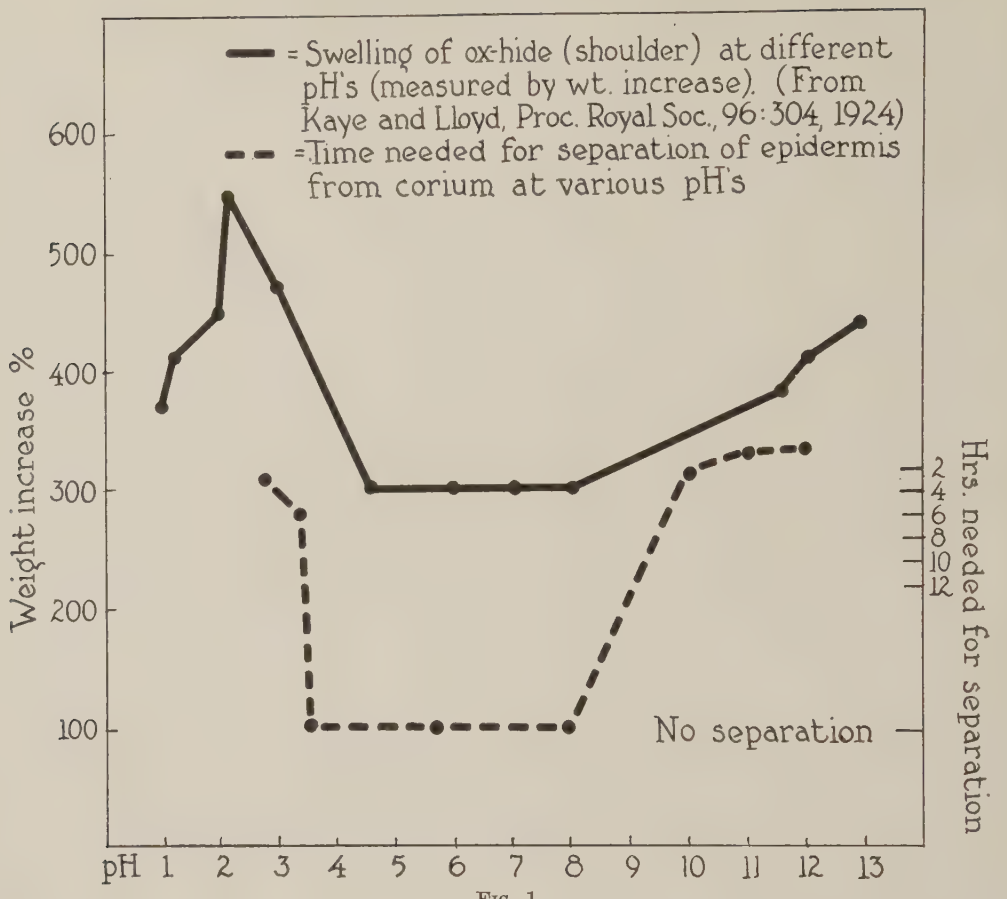

FIG. 1.

cohesive and adhesive properties,[7] an attempt was made to correlate the degree of swelling of the corium to the time necessary for separation of the epidermis. Two millimeter cubes of normal human skin were immersed in various solutions and the time necessary for removability of the epidermis by forceps noted. In acetic acid and sodium hydroxide at various pHs the ease of epidermal separation closely followed the swelling curves for collagen and gelatin. (Fig. 1). Maximum swelling and minimum time of separation occurred at the isoelectric point of collagen (pH 5). On either side of the isoelectric point the depressive effect of 2N salt solutions at neutral pH on swelling and at the same time on the ease of epidermal separation was evident. In neutral salts separation of the epidermis was promoted in the same order that these salts promote the swelling of gelatin (Hofmeister series). 2N solutions of sodium thiocyanate removed the epidermis in 5 minutes; sodium iodide in 14 minutes; sodium chloride in 6 hours and sodium acetate, sulfate and citrate not at all. The latter salts shrink the corium.

Loosening of the epidermis by acids, bases, or heat could be reversed by subsequent immersal of the skin into salt solutions with shrinking effect; there was a solid re-attachment for about 16 hours. Anions that promote swelling facilitated subsequent separation by heat, whereas ions with shrinking properties inhibited heat separation.

Discussion. Swelling of hydrophilic colloids decreases their cohesive and adhesive properties.[7] In human skin the diminution of adhesion between epidermis and corium by swelling is more spectacular than the de-

[7] Wood, T. B., and Hardy, W. B., *Proc. Royal Soc.*, London, 1909, **81**, 38.

crease of cohesion within the collagen. Should the reticular and elastic fibres be involved in epidermal-dermal adherence[2] the great swelling pressure of collagen would be sufficient to rupture these. It is concluded that swelling of collagen in the upper corium may lead to blister formation.

Summary. The ease of separation of the epidermis from the corium by acids, bases, and salt solutions parallels the swelling of collagen in these agents. Separation due to swelling agents can be reversed by shrinking agents. The adherence of normal epidermis to the corium is increased by shrinkage of the collagen.

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Distribution of Cholesterol, Cholesterol Esters and Phospholipid Phosphorus in Normal Blood.

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Many observations indicate that not only the absolute amounts of the different lipids, but also the ratios of these lipids to each other in the cells and the surrounding medium are important in physiological and pathological conditions. The most easily accessible system for the study of these ratios is blood. Much work has been done on changes in the individual lipid components in different diseases, but only a few papers deal with their interrelationships. Recently Peters and Man[1] studied the interrelations of serum lipids in normal persons and in patients with different diseases. The study of cell lipids, however, has been comparatively neglected so far, perhaps because the changes that occur there are relatively small when compared to the changes that take place in plasma lipids. In animal experiments (unpublished) we observed that under the influence of different factors the changes in the cell lipids were frequently opposite in direction to those in the plasma lipids. It occurred to us that these changes, although small, might still be significant, and it seemed advisable to investigate under different conditions the cholesterol, cholesterol ester, and phospholipid phosphorus content of both blood cells and plasma, simultaneously. To obtain a baseline for further observations we first determined the various lipid values on a group of normal individuals.

Material and Methods of Analysis. The observations here presented were made on 2 groups of patients. The first group consisted of 10 healthy males and 10 healthy females whose ages ranged from 19 to 35. The second group was made up of 20 surgical patients with no diseases known to affect lipid metabolism. This number was equally divided between the 2 sexes, and their ages were between 70 and 90 years. Two patients whose plasma cholesterol values, (334 mg % and 86 mg % respectively), differed from the mean by more than twice the standard deviation* were excluded and replaced by 2 others.

1 Peters, J. P., and Man, E. B., *J. Clin. Invest.*, 1943, **22**, 707, 715.

* The standard deviation was calculated by the help of the formula $SD = \sqrt{\frac{\Sigma d^2}{n-1}}$

The standard error can be calculated from this formula as follows: $SE = \frac{SD}{\sqrt{n}}$

Results were considered as probably significant where $m_1 - m_2 > 2\sqrt{SE_1^2 - SE_2^2}$.

TABLE I.
Mean Values and Standard Deviations of the Different Lipid Factors in the Young, Old, and Combined Age Groups.

Group	No.	Total cholesterol mg %		Cholesterol esters mg %		Phospholipid P mg %	
		Cells	Plasma	Cells	Plasma	Cells	Plasma
Young group, 20-35 yrs	20	171.0 ± 30.7	186.2 ± 25.9	18.4 ± 24.3	128.7 ± 16.3	13.6 ± 1.3	9.1 ± 1.1
Old group, 70-90 yrs	20	175.0 ± 25.0	199.1 ± 43.0	11.7 ± 15.0	129.8 ± 31.6	14.7 ± 1.1	9.0 ± 1.3
Combined groups	40	173.0 ± 27.6	192.7 ± 35.5	15.3 ± 20.3	129.3 ± 25.0	14.1 ± 1.4	9.0 ± 1.2

Blood was withdrawn from one of the cubital veins in the postabsorptive state (12 to 14 hours after the last meal) and was mixed with heparin. Within 15 minutes after the blood sample was taken the hematocrit value was determined, 5 cc of blood was prepared for extraction,[2] and the remainder of the blood was centrifuged to separate plasma and cells. Five cc of the separated plasma was also extracted. Aliquot amounts of the extracts were used for the determination of the different lipid fractions. Cholesterol was determined according to Bloor,[3] cholesterol esters according to Bloor and Knudson,[4] and phospholipid phosphorus according to Bloor[5] with the modification of Fiske and Subbarow.[6] The lipid fractions were determined in whole blood and plasma and the cell values were calculated from these and the hematocrit values. The calculated cell values were more consistent in our hands than the direct determinations on the centrifuged cells. The parallel values obtained by calculation checked well, whereas the direct determinations gave consistently lower figures, with poorly checking parallels. This was undoubtedly due to the fact that the addition of cells to the alcohol ether mixture always resulted in a coarse precipitate which had a tendency to clump further on heating. This experience is in contradistinction to the results of Boyd[7] who found that the experimental error was greater in indirect than in direct determinations. On the other hand, Man and Gildea[2] found that the Bloor's method of extraction, used by us, gave higher values, and checked better with their own results than Boyd's method.

Results. Our findings are summarized in Table I. All values are given in mg %. In order to obtain insight into the interrelationships of the various lipids different ratios

[2] Man, E. B., and Gildea, E. F., *J. Biol. Chem.*, 1937, **122**, 77.

[3] Bloor, W. R., *J. Biol. Chem.*, 1916, **24**, 227.

[4] Bloor, W. R., and Knudson, A., *J. Biol. Chem.*, 1916, **27**, 107.

[5] Bloor, W. R., *J. Biol. Chem.*, 1918, **36**, 33.

[6] Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, **66**, 375.

[7] Boyd, E. M., *J. Lab. Clin. Med.*, 1936, **22**, 237.

TABLE II.
Mean Values and Standard Deviations of the Different Lipid Ratios.

	Cholesterol esters	Cell cholesterol	Cell phospholipid P	Plasma cholesterol	Cell cholesterol
No. of subjects	Total cholesterol in plasma	Plasma cholesterol	Plasma phospholipid P	Plasma phospholipid P	Cell phospholipid P
40	0.68 ± 0.06	0.94 ± 0.26	1.60 ± 0.29	21.30 ± 2.60	12.32 ± 2.24

were calculated. These are presented in Table II. Due to the amount of chemical work involved in these determinations, relatively small groups were employed. Notwithstanding this, our values, and not only the means, but also the standard deviations, are in close agreement with the data of Peters and Man,[1] obtained on a rather large group of patients. Contrary to expectations, the differences found between corresponding values of the 2 age groups were very slight. The cholesterol ester/total cholesterol ratio was found to be somewhat lower and the coefficient of variation of the individual ratios somewhat greater than by other workers.[8,9] This slight difference can be explained by the different methods used.

As indicated by the coefficient of variation, the phospholipid values were more constant than the cholesterol values. Of the various ratios calculated, the ratio of the plasma cholesterol/plasma phospholipid phosphorus was found to be the most constant.

Comment. Partos and Hertzog[10] were perhaps the first to point out the importance of the simultaneous determination of the different lipids in red cells and plasma. Unfortunately their lipid values differ greatly from the generally accepted figures. Consequently little importance can be attached to the deductions made on the basis of their findings. On the other hand, enough experimental evidence was adduced by other workers with regard to the antagonistic effect of different lipids on the various physicochemical and biological mechanisms to warrant further investigation along the lines suggested. Differences exist in the influence exerted on the various processes not only between phospholipids and cholesterol, but also between the different members of the phospholipid group and between cholesterol esters and cholesterol. At this time we only want to mention a few of these antagonisms to point out the importance of further investigations in this direction.

Lecithin promotes,[11] free cholesterol inhibits cobra venom hemolysis.[12] Cholesterol esters, however, have no protective effect on hemolysis.[13] Cholesterol protects mammals against convulsions caused by tetanus toxin; lecithin increases sensitivity towards it.[13] Cholesterol and lecithin have an opposite effect on experimental epilepsy.[14] The intraperitoneal administration of cholesterol increases the depth and length of anesthesia in rabbits and mice.[15] Lecithin has no such effect. Similarly cholesterol decreases phagocytosis both *in vivo* and *in vitro*. Whereas lecithin has little or no effect on phagocytosis, it can neutralize the influence of cholesterol on it.

The choice of some of the ratios presented in Table II might seem to require explanation. The importance of at least 2 of these ratios is well known, however. The diagnostic significance of the cholesterol ester/total cholesterol ratio in plasma needs no further explanation. The constancy of the plasma cholesterol/plasma phospholipid phosphorus ratio has been recently pointed out.[1] In a subsequent paper the interpretation of some other ratios will be discussed.

8 Sperry, W. M., *J. Biol. Chem.*, 1936, **114**, 125.

9 Brun, G., H. K. Lewis Co., London, 1940.

10 Partos, A., and Hertzog, A., *Z. f. d. ges. exp. Med.*, 1932, **84**, 374.

11 Kyes, P., *Berliner Klin. Wchnschrft.*, 1902, **39**, 886 and 918.

12 Ransom, F., *Dtsch. med Wchnschrft.*, 1901, **27**, 194.

13 Degkwitz, R., *Ergebnisse der Physiol.*, 1932, **32**, 821.

14 Aird, R. B., and Gurchot, C. L., *Arch. Neur. and Psych.*, 1939, **42**, 491.

15 Foldes, F. F., and Beecher, H. K., *J. Pharm. Exp. Therap.*, 1943, **78**, 276.

The fact that there is practically no difference in the lipid values and their ratios in the young and old age group seems to be significant. This finding is in contradiction to some earlier views and speculations.[16] It compares favorably, however, with the finding that the plasma cholesterol and cholesterol ester values of infants are practically the same as those of adults.[17]

This constancy of the lipid values in different age groups indicates that even small changes, if uniformly encountered under pathological conditions, might prove to be significant.

Summary. 1. Total cholesterol, cholesterol esters, and phospholipid phosphorus were determined simultaneously in blood cells and plasma on 20 young healthy adults and 20 old patients with no known disorder of lipid metabolism. 2. Practically no difference was found in the lipid values of the 2 age groups. 3. Various ratios have been calculated from the lipid values determined. 4. The possible importance of similar studies under pathological conditions is pointed out.

16 Parhon, C. J., and Parhon, M., *Compt. Rend. soc. biol.*, 1923, **88**, 231.

17 Hodges, R. G., Sperry, W. M., and Andersen, O. H., *Am. J. Dis. Child.*, 1943, **65**, 858.

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Distribution of Cholesterol, Cholesterol Esters and Phospholipid Phosphorus in Blood in Thyroid Disease.

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That changes of the plasma cholesterol level accompany thyroid diseases has been reported repeatedly.[1,2] The variations of the phospholipid phosphorus values have been investigated less frequently.[3,4] The most extensive recent study on this subject is that of Peters *et al.*[2] With the exception of a brief comment by Boyd,[5] no data have been published on the changes of the blood cell lipids in thyroid disease.

In a previous paper the distribution of some lipid values in blood cells and plasma of normal persons was presented.[6] In the following, the results of similar studies made on hypo- and hyperthyroid patients before and after adequate clinical treatment are reported, and an effort is made to correlate our findings with a new concept of the pathological chemistry of thyroid disease.

Material and Methods of Analysis. The studies were carried out on unselected patients of the Thyroid Clinic of the Massachusetts General Hospital. The only discrimination made was that mild cases of hyperthyroidism (basal metabolic rate below +30%) were excluded.

The hypothyroid group consisted of 7 patients, 6 of them female, one male. The ages of the patients ranged from 41 to 69 years. The basal metabolic rate was between +3% and —40% before, and between +11% and —13% after treatment. None of the patients received any form of thyroid medication within 6 months of the study.

1 Hurxthal, L. M., *Arch. Int. Med.*, 1933, **51**, 22; 1933, **52**, 86; 1934, **53**, 762.

2 Peters, J. P., and Man, E. B., *J. Clin. Invest.*, 1943, **22**, 715.

3 Gildea, E. F., Man, E. B., and Peters, J. P., *J. Clin. Invest.*, 1939, **18**, 739; Man, E. B., Gildea, E. F., and Peters, J. P., *J. Clin. Invest.*, 1940, **19**, 43.

4 Boyd, E. M., and Connell, W. F., *Quart. J. Med.*, 1939, **8**, 41.

5 Boyd, E. M., and Connell, W. F., *Quart. J. Med.*, 1936, **5**, 455.

6 Foldes, F. F., and Murphy, A. J., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 215.

Blood was taken for examination before, several times in the course of, and after adequate clinical treatment. The thyroid hormone was administered either in the form of thyreoglobulin or thyroid extract.

The hyperthyroid group comprised 12 patients, only 10 of whom were studied after treatment. Four of the patients were males and 8 females. The basal metabolic rates ranged from +52% to +33% before, and from +18% to —22% after treatment. Eight of the 10 cases followed received surgical treatment, and 2 were treated with radioactive iodine. Six of the surgical cases were prepared for operation with thiouracil, and 2 with potassium iodide. Lipids were determined before, in the course of, and after treatment. To save space, only the values determined before and after treatment will be presented in both the hypo- and the hyperthyroid group. The methods of analysis have been described.[6]

Results. The results of the analyses of the cell plasma lipid values are presented in Table I, where it can be seen that, with the exception of the cell total cholesterol and the cell phospholipid phosphorus, the lipid values of the untreated hypothyroid group were significantly increased. The increase of plasma total cholesterol was greater (over 100%) than that of plasma phospholipid phosphorus (less than 60%) and the relative changes of the cholesterol esters of cells and plasma were even more pronounced. After treatment the lipid values returned nearly to normal with the exception that the cell cholesterol esters became somewhat lower and the plasma cholesterol esters remained somewhat higher than the comparable values of the normal controls.

Similarly marked differences were found in all but one of the lipid ratios of untreated hypothyroid patients (Table II). The only ratio which showed no significant difference was the cell total cholesterol/cell phospholipid phosphorus ratio. The cholesterol ester/total cholesterol ratio and the total cholesterol/phospholipid phosphorus ratios in plasma were found to be increased, the cell cholesterol/plasma cholesterol and the cell phospholipid phosphorus/plasma phospholipid

TABLE I.

Mean Values and Standard Deviations of the Different Lipid Factors in Hypo- and Hyperthyroid Patients Before and After Treatment. Figures Marked with Asterisk Are Statistically Significant as Compared to Normal Values.

Group	No. of cases	Total cholesterol		Cholesterol esters		Phospholipid P	
		Cells, mg %	Plasma, mg %	Cells, mg %	Plasma, mg %	Cells, mg %	Plasma, mg %
Hypothyroid patients							
Before treatment	7	198.8 ± 44.4	414.1 ± 79.1*	88.4 ± 49.0*	296.0 ± 43.5*	14.6 ± 1.5	14.3 ± 1.7*
After treatment	7	189.3 ± 27.7	213.8 ± 32.1	3.1 ± 7.7*	152.0 ± 15.1*	13.7 ± 2.7	8.7 ± 1.8
Hyperthyroid patients							
Before treatment	12	172.6 ± 31.0	156.2 ± 78.0	10.0 ± 16.5	98.2 ± 38.0*	14.5 ± 2.0	7.5 ± 2.1*
After treatment	10	184.8 ± 50.7	242.2 ± 50.9*	10.0 ± 14.0	162.5 ± 40.6*	15.1 ± 1.0*	10.3 ± 2.2
Normal	40	173.0 ± 27.6	192 ± 35.5	15.3 ± 20.3	129.3 ± 25.0	14.1 ± 1.4	9.0 ± 1.2

TABLE II.

Mean Values and Standard Deviations of the Different Lipid Ratios in Hypo- and Hyperthyroid Patients Before and After Treatment. Figures Marked with Asterisk are Statistically Significant as Compared to Normal Values.

Group		No.	Cholesterol esters: Total cholesterol in plasma	Cell cholesterol: Plasma cholesterol	Cell phospholipid P: Plasma phospholipid P	Plasma cholesterol: Plasma phospholipid P	Cell cholesterol: Cell phospholipid P
Hypothyroid patients	Before treatment	7	0.73 ± 0.06*	0.50 ± 0.22*	1.04 ± 0.13*	28.68 ± 1.51*	13.62 ± 3.08
	After treatment	7	0.72 ± 0.06	0.93 ± 0.30	1.65 ± 0.77	24.86 ± 2.22*	13.44 ± 1.51
Hyperthyroid patients	Before treatment	12	0.62 ± 0.33	1.26 ± 0.58	2.05 ± 0.49*	20.96 ± 4.00	12.18 ± 1.90
	After treatment	10	0.69 ± 0.05	0.78 ± 0.21	1.52 ± 0.39	23.65 ± 4.20*	12.25 ± 2.60
Normals		40	0.68 ± 0.06	0.94 ± 0.26	1.60 ± 0.29	21.30 ± 2.60	12.30 ± 2.24

phosphorus ratios were found to be decreased, as compared to normals. After treatment all ratios changed towards normal. Only the total cholesterol/phospholipid phosphorus ratio of the plasma remained somewhat elevated.

The changes found in the mean values of the different lipid factors of the untreated hyperthyroid group are less marked, and the standard deviations are relatively greater than in the untreated hypothyroid group (Table I). Consequently, statistically significant differences can only be found in the values of the plasma cholesterol esters and the plasma phospholipid phosphorus. Following treatment of the hyperthyroid patients all lipid values increased and with the exception of the cell cholesterol esters their mean values became higher than the corresponding values of the normal control group. The increase of the plasma cholesterol, plasma cholesterol ester, and cell phospholipid phosphorus was great enough to be statistically significant.

The situation is much the same with regard to the different lipid ratios (Table II). Before treatment, the changes are opposite in direction to those found in the hypothyroid group. But owing to the fact that the differences of the mean values are less, and the coefficients of variation are greater, only the cell phospholipid phosphorus/plasma phospholipid phosphorus ratio differs significantly from the normal. After treatment the ratios change toward normal and in some instances (cell cholesterol/plasma cholesterol, cell phospholipid phosphorus plasma/phospholipid phosphorus, plasma cholesterol/plasma phospholipid phosphorus ratios) even overshoot in the direction of the hypothyroid values.

Discussion. Recently Hoffmann and Hoffmann[7] in a stimulating monograph advanced an entirely new concept of the pathological chemistry of hyperthyroidism, with which they present considerable evidence both from their own experiments and from the results of other workers. Since our clinical data seem to be in agreement with some of their

[7] Hoffmann, F., and Hoffmann, E. J. de, *Public. del instit. fisiol.*, Universidad de Chile, 1943.

views, we should like to summarize, in the following paragraph, the main points of their theory before discussing our findings.

They believe that due to the action of certain enzymes (lecithinases), continuous breakdown of phospholipids occurs in the organism. One of these breakdown products, lysolecithin,[8] has marked biological activity. It plays an important role in the metabolism of the central nervous system; it influences cell permeability for certain substances, and if present in increased concentration, it exerts toxic effects on various organs. These breakdown products can be demonstrated in the blood in increased amounts after stimulation of the cervical sympathetic trunk, the vagus, or sciatic nerve. Under normal conditions the toxic effects of lysolecithin are neutralized by various sterols, mainly cholesterol and the steroid hormones of the adrenals, perhaps also of the gonads. The thyroid hormone has a stimulating effect on lysolecithin formation. Consequently, in hyperthyroidism, where an excess of thyroid hormone is produced, the lysolecithin formation is also increased. The organism tries to counteract the toxic effect of lysolecithin by mobilizing its sterol reserves. As long as these reserves are ample the toxic manifestations of hyperthyroidism are kept under control, but when the sterol reserves have been exhausted the toxic symptoms become manifest.

The changes of the blood lipids that other workers, as well as ourselves, have found in thyroid diseases are at least not at variance with these assumptions. Table I shows that in hypothyroidism both the plasma cholesterol and plasma phospholipid phosphorus are increased. The relative increase of the plasma cholesterol is greater than that of the plasma phospholipid phosphorus. This finding, as well as the marked increase of the plasma cholesterol/phospholipid phosphorus ratio are in agreement with the findings of Peters *et al.*[2] If the thesis that the decomposition of lecithin is regulated by the thyroid hormone is accepted, it seems logical that with decreased thyroid activity the breakdown of lecithin should be curtailed and the plasma phospholipid phosphorus level should rise. Moreover, since the available sterols (especially cholesterol) are not used up for the neutralization of lysolecithin, the accumulation of cholesterol in plasma can also be considered as satisfactorily explained.

After adequate treatment with thyroid hormone, conditions change in the direction of normal. Under the stimulating effect of the hormone the decomposition of phospholipids returns to normal, cholesterol is used up in increased amounts to neutralize the lysolecithin formed, and consequently both the cholesterol and phospholipid levels become lower.

Similar considerations might satisfactorily explain the chain of events in hyperthyroidism. In this condition the increased amount of thyroid hormone might cause increased phospholipid breakdown and result in increased lysolecithin formation. The organism tries to neutralize the toxic lysolecithin by mobilizing its sterol reserves and probably also by increasing the synthesis of cholesterol and steroid hormones. It can be assumed that as long as this compensatory mechanism is able to keep pace with the increased lysolecithin formation the patients are more or less compensated from the clinical point of view. The exhaustion of this compensating mechanism results in the rapid deterioration of the patient's condition and in extreme cases it might be manifested in the form of thyroid crisis. In agreement with this assumption the most constant change encountered in the blood lipid values of hyperthyroidism was the decrease of the plasma phospholipid phosphorus. The average plasma cholesterol level of 12 cases was also decreased, but as the relatively high coefficient of variation indicates, this decrease was not distributed evenly over all the cases. The same was found to be true for the plasma cholesterol/phospholipid phosphorus ratio. Following clinical treatment the lipid values not only return to normal, but even overshoot in the direction of the hypothyroid levels. The reason for this is undoubtedly the fact that, in contrast to hypothyroid pa-

8 Feldberg, W., *Ann. Rev. Physiol.*, 1941, **3**, 671.

tients, hyperthyroid patients have to be overtreated in order to obtain best clinical results. That this is so is indicated by the fact that after adequate surgery, (especially after thiouracil preparation), the basal metabolic rate is usually lower than normal and values of —15% to —20% are not rare.

The blood lipid changes became normal under iodine or thiouracil preparation even before surgery. The same was found to be true in 2 patients who were medically treated with radioactive iodine.

The hitherto unexplained beneficial effect of KI in hyperthyroidism might perhaps also be explained by a primary effect on lipids. It was found that KI administration caused a further elevation of the plasma cholesterol level in hypercholestermic rabbits.[9,10]

Both histological[11–14] and functional studies[15–17] have established the existence of liver involvement in hyperthyroidism. It is also generally accepted[18] that in liver diseases the plasma ester cholesterol/total cholesterol ratio is decreased. In agreement with these facts the average plasma cholesterol ester/total cholesterol ratio in our 12 cases was found to be 0.62 ± 0.33 before treatment as compared to the normal average of 0.68 ± 0.06. As the large coefficient of variation indicates, the variation of the individual ratios, just as in the case of the other lipids values in hyperthyroidism, was very large.* It has been shown by some workers that in general the plasma cholesterol level is inversely proportional to the clinical toxicity of hyperthyroid patients and the plasma cholesterol level increases after treatment.[2,19] We have now found that in our small series the plasma ester cholesterol/total cholesterol ratio behaves similarly: the more toxic the patient, the lower the ratio. Following medical or surgical treatment, not only the ratio but also its coefficient of variation returns to normal.

We should like to emphasize that the Hoffmann hypothesis can by no means be considered as proven. On the other hand, there is enough suggestive evidence in its support to warrant further clinical and pharmacological studies. An example of its possible usefulness is that the hitherto unexplained blood lipid changes of thyroid disease can be interpreted by it in a way which is in agreement with the already known antagonistic properties of cholesterol and lecithin.[20]

Summary. 1. The distribution of total cholesterol, cholesterol esters, and phospholipid phosphorus was studied in the blood cells and plasma of thyroid patients. 2. The plasma cholesterol, plasma cholesterol ester, and plasma phospholipid phosphorus were found to be significantly increased in hypothyroid patients. 3. The changes of the plasma lipids were less consistent in hyperthyroidism. The plasma phospholipid phosphorus alone showed a significant decrease. 4. The cell lipid values were found relatively constant both in hypo- and hyperthyroidism. 5. Definite changes were also found in the different lipid ratios in hypothyroidism. The plasma cholesterol ester/total cholesterol ratio and the plasma cholesterol/plasma phospholipid phosphorus ratio were significantly increased. The cell cholesterol/plasma cholesterol and the cell phospholipid phosphorus/plasma phospholipid phosphorus ratios were significantly decreased. Only the cell cholesterol/cell phospholipid phosphorus

9 Rosenthal, S. R., *Arch. Path.*, 1934, **18**, 827.

10 Page, I. H., and Bernhard, W. G., *Arch. Path.*, 1935, **19**, 530.

11 Bert, J. E., and Goldburgh, H. L., *Internat. Clinic.*, 1941, **4**, 126.

12 Beaver, D. C., and Pemberton, J. DeJ., *Ann. Int. Med.*, 1933, **7**, 687.

13 Weller, C. V., *Ann. Int. Med.*, 1933, **7**, 543.

14 Lord, J. W., and Andrus, W. deW., *Arch. Surg.*, 1941, **42**, 643.

15 Haines, S. F., Magath, T. B., and Power, M. H., *Ann. Int. Med.*, 1941, **14**, 1225.

16 Smith, M. C., Johndahl, W., and Ochsner, A., *Surg. Gyn. Obs.*, 1942, **74**, 1083.

17 Mills, F. H., *Med. J. of Australia*, 1942, **1**, 195.

18 Man, E. B., Kartin, B. L., Durlacher, S. H., and Peters, J. P., *J. Clin. Invest.*, 1945, **24**, 623.

* In contrast to our findings, Peters *et al.* found no changes in the plasma cholesterol partition of 9 thyroid patients.

19 Cutting, W. C., Rytand, O. A., and Tainter, L. M., *J. Clin. Invest.*, 1934, **13**, 574.

20 Degkwitz, R., *Ergeb. Physiol.*, 1931, **32**, 821.

ratio remained unchanged. 6. In hyperthyroidism the change in the ratios was again less constant. The only significant change was the increase of the cell phospholipid phosphorus/plasma phospholipid phosphorus ratio. 7. Both the lipid values and the various lipid ratios returned towards normal following adequate treatment.

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Relation Between Metabolic Activity and Cyanide Inhibition in *Pelomyxa carolinensis* Wilson.*

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Many investigations have been made on various animal species in ascertaining the presence or absence of a cytochrome-cytochrome oxidase system in living cells. In recent years numerous reports have been published in which protozoan cells have been used as experimental organisms. Some of these unicellular forms make excellent material for this type of investigation, and all of them are sensitive to cyanides, although, formerly, there was some doubt about this, especially in reference to ciliates.

Lund,[1] Shoup and Boykin,[2] and Gerard and Hyman[3] maintained that *Paramecium caudatum* was non-sensitive to HCN. Peters[4] claimed that respiration in *Colpidium colpoda* was not inhibited by potassium cyanide and Pitts[5] working with *Colpidium campylum* and Lwoff[6] with *Glaucoma piriformis* found that there was an initial inhibition when these forms were exposed to cyanide, but that oxygen consumption soon increased to normal. Hall,[7] however, definitely proved that cyanide inhibits respiration in *Colpidium campylum* and Baker and Baumberger[8] found the same to be true for *Tetrahymena geleii.*

The apparent non-sensitivity of *Paramecium* to cyanide was questioned by Hyman in unpublished data referred to by Child[9] and Pace.[10] Saito and Tamiya[11] found cytochrome in *Paramecium,* which led to the supposition that respiration in *Paramecium* might depend, at least partly, upon cytochrome-cytochrome oxidase. Since that time, Boell[12] working with *Paramecium calkinsi,* Pace[10] with *P. caudatum* and *P. aurelia;* and Clark[13] with *P. caudatum* found that oxygen consumption of these species is inhibited by cyanide. Evidence has now accumulated to such an extent that the sensitivity of ciliates to cyanide is no longer questioned.

According to Pace,[10] sensitivity to cyanide in *P. aurelia* and *P. caudatum* is apparently dependent upon the degree of carbohydrate metabolism. He finds that inhibition is much more pronounced when glucose is pres-

* These investigations were partly supported by a grant-in-aid obtained from the Society of Sigma Xi.

1 Lund, E. J., *Am. J. Physiol.,* 1918, **45**, 365.

2 Shoup, C. S., and Boykin, J. T., *J. Gen. Physiol.,* 1931, **15**, 107.

3 Gerard, R. W., and Hyman, L. H., *Am. J. Physiol.,* 1931, **97**, 524.

4 Peters, R. A., *J. Physiol.,* 1929, **68**, 11.

5 Pitts, R. F., PROC. SOC. EXP. BIOL. AND MED., 1932, **29**, 542.

6 Lwoff, M., *C. R. Soc. Biol.,* Paris, 1934, **115**, 237.

7 Hall, R. H., *Physiol. Zool.,* 1941, **14**, 193.

8 Baker, E. G. S., and Baumberger, J. P., *J. Cell. Comp. Physiol.,* 1941, **17**, 285.

9 Child, C. M., *Patterns and Problems of Development,* 1941, 811.

10 Pace, D. M., *Biol. Bull.,* 1945, **89**, 76.

11 Saito, T., and Tamiya, H., *Cytologia,* 1937, Fujii Jubilee Volume, 1133.

12 Boell, E. J., *Anat. Rec.,* 1942, **84**, 493.

13 Clark, A. M., *Aust. J. Exp. Biol. and Med.,* 1945, **23**, 317.

TABLE I.

Buffered Culture Solution Used in Growing and Maintaining *Pelomyxa carolinensis* and Its Food Organism, *Paramecium caudatum*.

$K_2HPO_4 \cdot 3H_2O$	65.5 mg
$NaH_2PO_4 \cdot H_2O$	40.0 "
$CaCl_2$	100.0 "
$MgCl_2$	2.0 "
H_2O (redistilled) to	1000 ml

ent in the test media, than otherwise. This, and the fact that starved specimens are non-sensitive whereas respiration in "well-fed" specimens is inhibited by cyanide, indicates a possible connection between the cytochrome oxidase system and degree of metabolism (carbohydrate metabolism) in *Paramecium*.

The following report is the outcome of further investigations based on this supposition and is concerned with the following problems: (1) the relation between temperature change and respiratory quotient, (2) respiratory inhibition by cyanide as influenced by changes in temperature, and then (3) the cytochrome-cytochrome oxidase mechanism and carbohydrate metabolism in *Pelomyxa carolinensis*.

Material and Methods. Pelomyxa carolinensis Wilson, the organism used throughout these investigations, has been cultured in this laboratory for the past 3 years. It was grown in a modification of the buffered culture solution used by Pace and Belda[14] and is given in Table I (hydrogen ion concentration = pH 6.8 to 7.0).

Pelomyxa reproduces and grows rapidly in this solution when fed *Paramecium* (usually *caudatum*) in large numbers.

The rates of oxygen consumption and carbon dioxide elimination were ascertained by means of a Barcroft-Warburg apparatus.

Hydrochloric acid (0.3 ml 3 N HCl) was placed in the onset of each of the manometer flasks to absorb any ammonia given off by the organisms (Specht[15]) and to be used in releasing bound carbon dioxide at the end of the experiment. An alkali solution (0.2 ml 1 N KOH) was placed in the inset of the manometer flasks employed to determine the rate of oxygen consumption. The so-called "direct method" was used for oxygen consumption as well as carbon dioxide elimination.

Low temperatures were maintained by means of an Aminco portable cooling unit equipped with a thermo-regulator.†

Results. I. *Effects of Temperature Upon Respiration and Respiratory Quotient.* A series of tests was conducted to ascertain the rate of oxygen consumption in *Pelomyxa carolinensis,* at temperatures ranging from 10° to 35°C. Temperatures of 40°C were not employed since Pace and Belda[14] had reported that 9 of the 12 tests which they performed at 40°C were terminated in the first hour due to death of the organisms, while the specimens in the remaining 3 tests survived only long enough to permit a single reading of the manometers.

The accuracy of the determination of the rate of oxygen consumption decreases directly with a decrease in temperature chiefly because the rate of metabolism decreases with a reduction in temperature (Pace and Belda[14]); therefore, proportionately larger numbers of test organisms were employed at lower temperatures, *i.e.*, as many as 800 organisms were used when the experiments were conducted at 10°C.

The required number of pelomyxae of average size was removed individually from the culture dishes with a capillary pipette, washed thoroughly with buffer solution and placed in 5 ml of the same buffer solution in the main compartment of 6 of the manometer flasks. These experiments were carried out in order to ascertain respiratory quotients of pelomyxae at 10°, 20°, 30° and 35°C.

It was noted that the test organisms at 10°C tended to assume a spherical and compact shape, coincidently with a decrease in the rate and amount of protoplasmic streaming. Conversely, with an increase in temperature (30° and 35°C), the organisms showed marked increase in both the rate of

[14] Pace, D. M., and Belda, W. H., *Biol. Bull.*, 1944, **86**, 146.

[15] Specht, H., *J. Cell. Comp. Physiol.*, 1934, **5**, 319.

† The purchase of a portable cooling unit was made possible by a grant-in-aid furnished by the University of Nebraska Research Council.

TABLE II.

Rate of Oxygen Consumption and Carbon Dioxide Production in *Pelomyxa carolinensis* at Different Temperatures. Average Volume of One Million Organisms, 35,145 mm³.

Temp., °C	Test No.	No. of organisms used	Duration of exp., hr	Avg O_2 consumption in mm³/hr/ million organisms	Avg CO_2 elimination in mm³/hr/ million organisms	Avg rate of O_2 consumption in mm³/hr/mm³ of cell substance	Avg rate of CO_2 elimination in mm³/hr/mm³ of cell substance
10	1	750	7	3866	1937		
	2 to 3	500	7	4453	1150		
	4 to 6	500	7½	2658	694	.070	.032
	7 to 9	800	17	1454			
	10 to 12	800	7	2199			
				Mean—2442	Mean—1119		
						R. Q. = .45	
20	1 to 6	200	6	4989	2597		
	7 to 12	200	7	6000	4410	.160	.119
	13 to 17	200	6	4660	4208		
	18 to 23	250	6	7167	6245		
				Mean—5594	Mean—4190		
						R. Q. = .75	
30	1 to 5	250	8	10166	8579		
	6 to 10	250	7	11145	10720	.332	.311
	11 to 13	250	8	13643	14147		
				Mean—11664	Mean—10920		
						R. Q. = .94	
35	1 to 3	150	7	24042	24601		
	4 to 6	150	7	34203	24391	.767	.673
	7 to 9	250	8	22668	21965		
				Mean—26971	Mean—23652		
						R. Q. = .88	

protoplasmic streaming and in the increased length and movement of pseudopodia, almost assuming stellate forms. Macroscopic (and occasionally, microscopic) examinations of the specimens immediately upon termination of the experiments, prior to the addition of the acid from the onset, showed that the specimens remained alive throughout the experiments. Organisms subjected to low temperatures were found to be in a compact and spherical shape; those at high temperatures were stretched out and formed pseudopods.

The results are given in Table II. They indicate that the respiratory quotient increases directly with temperature, along with rate of oxidation. Carbon dioxide elimination increases with an increase in temperature to a point where (at 30°C and at 35°C) the carbon dioxide output in some tests exceeds oxygen consumption.

II. *Effects of Potassium Cyanide on Respiration at Different Temperatures.* The effects of potassium cyanide upon both the structure and activity of *Pelomyxa* have already been determined by Pace and Belda.[16] They also ascertained the effects of potassium cyanide as well as other poisons on respiration in *Pelomyxa carolinensis*. However, nothing has appeared in the literature in reference to the possibility of different cyanide effects at different temperatures. Since the rate and possible type of metabolism varies with temperature, it seemed worthwhile to determine the effects of potassium cyanide at different temperatures.

Again, temperatures of 10°, 20°, 30° and 35°C were used. Potassium cyanide (10^{-4} M) was added to the culture solution in 3 of

[16] Pace, D. M., and Belda, W. H., *Biol. Bull.*, 1944, **87**, 138.

TABLE III.

Effect of Potassium Cyanide at Different Temperatures on Oxygen Consumption in *Pelomyxa carolinensis*. Average volume of One Million Organisms, 35,145 mm³.

Temp., °C	Test No.	No. organisms used	Duration of exp., hr	Avg O_2 consumption in mm³/hr/million organisms	Avg O_2 consumption KCN (10^{-4} M) in mm³/hr/million organisms	Avg rate of O_2 consumption in mm³/hr per mm³ of cell substance	Avg rate of O_2 consumption KCN (10^{-4} M) in mm³/hr/mm³ of cell substance	% inhibition
10	1 to 3	500	8	1644	898			
	4 to 6	500	8	1183	848	.052	.024	54
	7 to 8	500	8	2033	712			
				Mean—1823	Mean—845			
20	1 to 2	500	5	7725	4070			
	3 to 4	500	5	5572	1845	.209	.085	60
	5 to 7	500	6	8354	3068			
				Mean—7380	Mean—3000			
30	1 to 2	250	6	20780	4715			
	3 to 4	250	10	20610	6778	.589	.150	75
	5 to 6	250	6	20763	4346			
				Mean—20718	Mean—5280			
35	1 to 2	150	6	32416	2482			
	3 to 4	150	5	29520	4850	.928	.175	81
	5 to 6	150	6	35967	11210			
				Mean—32634	Mean—6180			

the manometer test-flasks; the remaining 3 were used as controls.

Many of the test organisms which had been subjected to cyanide action at a temperature of 35°C during the course of the experiment were found to be, upon termination of the test, fragile and susceptible to rupture upon handling by means of a transfer pipette. Following rupture, the cytoplasmic mass exuded into the surrounding solution forming a slightly cloudy mixture. However, specimens in contact with cyanide at lower temperatures of 30°, 20° and 10°C did not disintegrate upon handling.

Comparisons between the rates of oxygen consumption in the absence of cyanide and in the presence of 10^{-4} M potassium cyanide are presented in Table III.

It is obvious from these results, that the degree of inhibition of oxygen consumption by potassium cyanide increases directly with the temperature; 54% inhibition at 10°C to 81% at 35°C.

Discussion. As stated previously, Pace,[10] working with the effect of potassium cyanide on respiration in *Paramecium caudatum* and *Paramecium aurelia* noted that inhibition depends upon the degree of saturation of the respiratory system of the organisms with carbohydrate. These results, in general, were confirmed by Clark[13] for *Paramecium caudatum*. It is evident, therefore, that respiratory sensitivity to the action of cyanide is dependent in part upon the amount of carbohydrate material present in the cell. Keilin[17] proposed that the concentration of carbohydrate material within the cell is, more than any other factor, responsible for cellular sensitivity to cyanide. Commoner,[18] working with yeast, reported that the percentage of inhibition by cyanide is dependent upon the concentration of dextrose present within the cell. He noted also that, in the absence of dextrose, little or no respiratory inhibition occurred. Emerson[19] investigating cyanide-sensitivity with the alga *Chlorella*, Hall,[7] with *Colpidium campylum*, and Baker and Baumberger,[8] with *Tetrahymena geleii*, confirmed the above findings.

Pace and Belda[16] suggested that respiration in *Pelomyxa carolinensis* appeared to occur chiefly through a cytochrome-cytochrome oxidase system.

Results presented in Table II show that, evidently, metabolism is chiefly carbohydrate at higher temperatures since it was found that at 30°C, the R.Q. was nearly 1.0; that at 10°C, 0.45. Results shown in Table III upon the effects of cyanide on respiration, yield values which indicate that oxidative inhibition increases directly with an increase in temperature, in some instances approaching total inhibition. Thus, cyanides are much more effective when carbohydrate metabolism is apparently great, which, in turn, leads to the conclusion that in *Pelomyxa* the cytochrome-cytochrome oxidase type of respiratory system is concerned with carbohydrate metabolism. The greater the quantity of carbohydrate oxidized, the greater is the effect of cyanide.

Summary. Oxygen consumption and carbon dioxide elimination in *Pelomyxa carolinensis* was ascertained by means of a Barcroft-Warburg micro-respirometer. The total average oxygen consumption was found to be 2442 mm^3, 5594 mm^3, 11,664 mm^3 and 26,971 mm^3 per hour per million organisms (or 0.07, 0.16, 0.33, and 0.76 mm^3 per hour per cubic millimeter cell substance) at 10°, 20°, 30°, and 35°C, respectively.

The respiratory quotient varies directly with the temperature. It was found to be 0.45 at 10°C and 0.94 at 30°C. Consequently, carbohydrate metabolism is evidently greater at higher than at lower temperatures.

Respiration is inhibited by potassium cyanide (10^{-4} M) and the degree of inhibition varies directly with the temperature.

From the evidence presented, it can be concluded that a cytochrome-cytochrome oxidase system is the mechanism chiefly involved in oxidation of carbohydrate in *Pelomyxa* and that as carbohydrate metabolism is reduced in its extent, this mechanism becomes less and less active.

17 Keilin, D., *Ergeb. der Enzymforschung.*, 1932, **2**, 239.

18 Commoner, B., *J. Cell. Comp. Phys.*, 1939, **13**, 121.

19 Emerson, R., *J. Gen. Physiol.*, 1927, **10**, 469.

15428 P

Influence of Anticholinesterase Diisopropylfluorophosphate on Cardio-vascular and Respiratory Reflexes of Carotid Sinus Origin.

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In previous investigations we were able to show that the anticholinesterase prostigmine has no effect on the central transmission of the carotid sinus pressoceptive reflexes acting on the cardio-inhibitory, vasomotor and respiratory centers.[1] Recently O. Bodansky and A. Mazur[2] reported on the very marked anticholinesterase activity on blood and tissues of diisopropylfluorophosphate (D.F.P.).

The influence of the anticholinesterase D.F.P. on the respiratory, cardiac and the vasomotor reflexes induced by the carotid sinus presso-ceptors in the dog has been investigated.

Method. In the dog, under choralosane anesthesia, the efferent arteries of the carotid sinus were ligated, care being taken not to sever the pressoceptive innervation. The cephalic end of the common carotid artery was connected with a pressure device. By means of this technic, the hydrostatic pressure may be increased or decreased in the isolated presso-sensitive innervated carotid sinus. The cardio-vascular and respiratory reactions of the animal to the intracarotid sinus pressure changes were registered before and after injection of the anticholinesterase D.F.P. The cholinesterase activity of the blood was determined by means of the method described by A. L. Delaunois and H. Casier.[3]

Results. Intravenous injections of doses of D.F.P. (0.12-1 mg/kg) inhibiting completely the cholinesterase activity of the blood, do not stimulate the respiratory center and do not affect either the heart rate or the blood pressure. The cardio-vascular and respiratory reflexes induced by increase or decrease of the intracarotid sinus pressure are not affected by the administration of D.F.P. and the inhibition of the cholinesterase activity. The above results do not support the theory of a cholinergic transmission of the respiratory and cardio-vascular reflexes induced by means of a physiological stimulation of the carotid sinus pressoceptors.

[1] Heymans, C., Pannier, R., and Verbeke, R., *Thirtieth An. Meet. Fed. Am. Soc. f. exp. Biol.*, 1946.

[2] Bodansky, O., and Mazur, A., *Fed. Proc.*, 1946, **5**, 123.

[3] Delaunois, A. L., and Casier, H., *Experientia*, 1946, II, 2, 4.

15429

Growth of Influenza Virus in Eggs in the Presence of Bacterial Contamination and Streptomycin.*

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(Introduced by Chester S. Keefer).

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With a few possible exceptions viruses appear to be unaffected in their growth in living hosts in the presence of a wide variety of antibiotics.[1] In general one would ex-

* We are indebted to Dr. Chester S. Keefer for the supply of streptomycin, and to Miss Marjorie Jewell and Miss Carla Bryan for determining the streptomycin levels.

[1] Kramer, S. D., Geer, H. A., and Szobel, D. A., *J. Immunol.*, 1944, **49**, 273.

pect that the addition of a chemotherapeutic agent to virus-containing specimens with bacterial contamination would suppress bacterial growth without interfering with the proliferation of the virus. Although the influenza virus will grow readily in the allantoic or amniotic sac of the chick embryo in the presence of heavy bacterial contamination contained in unfiltered throat washings[2–4] the growth of virus is favored and the life of the embryo tends to be prolonged when penicillin is present.[3,5] Attempts to use zephiran[2,5,6] and sulfadiazine[5] as a means of controlling bacterial contamination in eggs were unsuccessful.

Streptomycin has been shown to have no observable adverse effect on the developing chick embryo or the proliferation of influenza virus in the allantoic sac.[7] It also prevents the growth of certain gram negative organisms in the chick embryo[8] and it has been shown to appear in the blood in small concentrations when applied to the surface of the chorioallantoic membrane.[9] In the present study, some of these observations have been confirmed and streptomycin has been further shown to be highly effective in preventing interference with the growth and recovery of influenza virus by the naturally occurring organisms present in unfiltered throat washings and feces.

Materials and Methods. Throat washings, stool suspensions and dilutions of virus were made with sterile brain broth containing 5% horse serum. Human group O cells were used in the virus titrations and for identification of virus by inhibition of red cell agglutination[10] with specific serum prepared in rabbits. All injections in eggs were made into the allantoic sac of 10- to 11-day white leghorn embryos with .1 cc volumes of various mixtures of streptomycin, contaminated material and virus. The eggs were incubated at 36°C to 38.5°C in a regular bacteriologic incubator. The PR8 strain of Influenza A virus and the Lee strain of Influenza B virus were diluted from infected allantoic fluids stored in sealed tubes at —60°C. As grown in this laboratory, the titer of Influenza A virus in the allantoic fluid after incubation for 48 hours, as measured by red cell agglutination,[10] was 1:64 to 1:128 and that for Influenza B virus was 1:8 to 1:16, occasionally 1:32. The streptomycin was prepared by Merck and Company (Lots No. 251 and 412) and was dissolved in distilled water to make a solution containing 100,000 units per cc. Assays of streptomycin in allantoic and amniotic fluids were carried out by an adaptation of the cup plate method in which a strain of staphylococcus was used as the test organism.[11]

Results. Injection of streptomycin and virus into the allantoic sac. Varying amounts of streptomycin were injected into the allantoic sac of chick embryos and, after 48 hours' incubation, a number of allantoic and amniotic fluids were assayed for streptomycin. The results are shown in Table I. Eggs No. 1 to 12 received streptomycin alone. All the embryos were alive at the end of 48 hours and had a normal appearance. Eggs No. 13 to 24 received a mixture of streptomycin and Influenza A virus in a final dilution of 10^{-7}. At the end of 48 hours the embryos were also alive and virus was present in each allantoic fluid in the usual titer. Eggs No. 25 to 36 were likewise injected with a mixture of streptomycin and Influenza B virus in a final dilution of 10^{-4}. All but 2 embryos (No. 25 and 32) were alive at the end of 48 hours and virus was again present in the usual titer.

2 Eaton, M. D., Corey, M., van Herick, W., and Meiklejohn, G., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 6.

3 Hirst, G. K., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 155.

4 Rickard, E. R., Thigpen, M., and Crowley, J. H., *J. Immunol.*, 1944, **49**, 263.

5 Rose, H. M., Milloy, E., and O'Neill, E., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 23.

6 Personnel of U. S. Naval Laboratory Research Unit No. 1, *Science*, 1942, **96**, 53.

7 Florman, A. L., Weiss, A. B., and Council, F. E., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 16.

8 Jones, D., Metzer, J. J., Schatz, A., and Waksman, S. A., *Science*, 1944, **100**, 103.

9 Lee, H. F., Stavitsky, A. B., and Lee, M. P., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 143.

10 Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.

11 Stebbins, R. B., and Robinson, H. J., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 255.

TABLE I.
Concentration of Streptomycin in the Allantoic and Amniotic Fluids 48 Hours After Injection of Streptomycin into the Allantoic Sac.

Units of streptomycin injected into allantoic sac								
2,500			250			50		
	Units per cc after 48 hr in			Units per cc after 48 hr in			Units per cc after 48 hr in	
Egg No.	Allantoic fluid	Amniotic fluid	Egg No.	Allantoic fluid	Amniotic fluid	Egg No.	Allantoic fluid	Amniotic fluid
1	172	10	5	33, 46		9	4.4	±
2		5	6	>26	5	10		1
3	216	10	7	>26	5	11	8	±
4		10, 11	8	14		12		±
13	400	0	17	17	0	21	4.7	0
14	66, 88*		18	20	0	22	0	
15	66, 88		19	14, 20		23	4.5	
16	>52, 120	0	20			24	4.4	
25	160		29	10, 16	1.1	33	3.4	±
26	90		30	16		34	3	±
27	90, 122	4, 5	31	16	4	35	5	±
28	90	8, 10	32			36	4.5	±

* The presence of 2 figures in a square indicates that duplicate assays were made.

These results indicate that the injection of 2,500 units or less of streptomycin into the allantoic sac of the 10- to 11-day chick embryo is without apparent adverse effects on the embryo and does not interfere with proliferation of Influenza A or B virus. Furthermore, with the largest amount of streptomycin used, highly bacteriostatic or bactericidal concentrations persist in the allantoic fluid for at least 48 hours, but only small amounts appear in the amniotic fluid.

Experiments with Throat Washings. To throat washings obtained from a healthy adult were added Influenza A virus to make a final dilution of 10^{-8} and streptomycin to make a final concentration of 2,500 units per cc. After standing for an hour,† 1.5 cc was mixed with .5 cc of a solution containing 100,000 units of streptomycin per cc. Each of 6 10-day eggs was injected intra-allantoically with .1 cc of this mixture. A second group of 6 eggs was injected with throat washings and virus with no streptomycin added. After 48 hours' incubation, all 6 eggs receiving virus and throat washings without streptomycin were grossly contaminated and no attempt was made to demonstrate influenza virus. Of the 6 eggs receiving the mixture containing streptomycin, 2 were dead and 4 were alive, no organisms were seen on smear and none grew on culture. All fluids from these 6 eggs gave agglutination of red cells in a titer of 1:64 or higher.

Streptomycin and Suspensions of Stool. A mixture of human stool in a final dilution of 1:50, Influenza A virus in a final dilution of 10^{-7} and streptomycin in a final concentration of 2,500 units per cc was allowed to stand at room temperature for one hour, after first removing the coarser stool particles by spinning at 1,500 r.p.m. for 5 minutes. To 1.5 cc of this mixture was added .5 cc of a solution of streptomycin containing 100,000 units per cc and .1 cc was then injected into a number of eggs. Each egg received, therefore, slightly more than 2,500 units of streptomycin in combination with stool suspension in a final dilution of approximately 1:66 and virus. After incubation for 48 hours, the allantoic fluid was removed from those embryos showing no gross evidence of contamination as indicated by marked cloudiness of the fluid, foul odor and, frequently, rupture of the yolk sac.

† This was done on the assumption that the streptomycin would act more effectively on the contaminating organisms.

Preliminary tests with virus-stool mixtures in the absence of streptomycin yielded grossly contaminated embryos in every instance. This was also true of eggs receiving virus-stool mixtures containing 10,000 units of penicillin per cc. Of 36 eggs injected with a mixture containing stool, virus and streptomycin, 6 embryos were grossly contaminated and were discarded. Of the remaining 30 embryos, 23 were dead but the embryos appeared intact, there was no odor, and the allantoic fluid was only faintly cloudy. The fluid was indistinguishable in appearance from that obtained from embryos in which influenza virus has grown without added contaminating material. The allantoic fluid of the 6 living embryos had a similar appearance. Smears of these 30 fluids stained by the Gram method showed no organisms. However, culture on blood agar under aerobic conditions was positive in 6 instances. Five of these fluids were obtained from dead embryos. Influenza virus was readily demonstrable by red cell agglutination in each of the 30 fluids and the titers varied between 1:64 and 1:128. Control tests done with allantoic fluid derived from 6 eggs injected with mixtures of streptomycin and stool suspension without added virus failed to show any agglutination of red cells by such fluids. All embryos in this group were alive at the end of 48 hours.

Influenza A virus, free from any demonstrable bacterial contamination either by aerobic or anaerobic culture, was readily obtained by Seitz filtration of virus-infected allantoic fluid from an embryo receiving an injection of Influenza A virus, stool suspension and streptomycin. The filtered virus produced typical pulmonary lesions following intranasal instillation in mice, grew well in the allantoic sac and failed to cause agglutination of red cells when mixed with serum prepared against Influenza A virus, but did cause agglutination when mixed with serum prepared against Influenza B virus.

The results of these studies made with mixtures of streptomycin, suspensions of human stool and Influenza A virus indicate that virus will grow readily in the allantoic sacs of 10- to 11-day chick embryos in the presence of fecal contamination. It is doubtful that all such virus-infected fluids are entirely free from bacterial contamination, but Seitz filtration of the allantoic fluid yielded Influenza A virus free from detectable bacterial contamination and indistinguishable in its behavior from the original virus. Frequent death of the embryos was observed in these experiments which could not be attributed either to the virus alone or to streptomycin. The failure of a streptomycin-stool mixture, without added virus, to kill chick embryos suggests that death in the presence of virus is due to the combination of virus and fecal contamination.

Conclusions. 1. Streptomycin appears to have no adverse effect on the 10- to 11-day chick embryo when injected into the allantoic sac.

2. Streptomycin is demonstrable in bacteriostatic or bactericidal concentrations in the allantoic fluid 48 hours after a single injection of 2,500 units into the allantoic sac. Only small amounts appear in the amniotic fluid.

3. Streptomycin in the concentrations used appeared to have no adverse effect on the growth of either Influenza A or B virus in the allantoic sac.

4. Streptomycin is highly effective in preventing gross contamination of allantoic fluids of chick embryos injected with unfiltered throat washings and suspensions of human stool.

5. Influenza A virus, when highly diluted and mixed with unfiltered throat washings or stool suspensions containing added streptomycin, can be readily recovered after growth in the allantoic sac of the developing chick embryo.

6. The results indicate that the loss of virus which may follow removal of contaminants by filtration may be avoided by the addition of streptomycin to unfiltered specimens derived from animals or man. After growth in the chick embryo, the virus may then be recovered free from contamination by filtration if necessary.

15430

Effect of Insulin, Insulin-Dextrose, and Water Diuresis on Metabolism of Isopropyl Alcohol.*†

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Since it is a reasonable assumption that the important toxic effect of isopropyl alcohol is on the central nervous system,[1] it follows that any therapeutic measure which may limit access of the alcohol to the nervous tissue could be effective in the prevention and treatment of isopropyl alcohol poisoning. Any procedure which is aimed at removal of the agent from the blood could achieve this objective. The present investigation deals with the influence of insulin, insulin plus dextrose, and water diuresis on the post-absorptive blood levels of isopropyl alcohol and acetone as observed in dogs.

Procedure. Isopropyl alcohol was administered intravenously to 3 female dogs in doses of 1 g per kg diluted to a 20% concentration in 0.9% sodium chloride solution. The rate of injection was kept constant at 0.25 cc per kg per minute so that the administration time was 25 minutes for each injection. One hour was allowed for equilibration and complete urine specimens, obtained by catheterization, and blood samples were taken hourly thereafter for 6 hours. Analysis for isopropyl alcohol was carried out by a colorimetric method adapted from a procedure described for ethyl alcohol.[2] Acetone was determined by the method of Greenberg and Lester.[3] All animal work was conducted in air-conditioned laboratories, hence environmental conditions were the same throughout.

After the control values were established for the 3 animals, the test dose of alcohol was repeated with one of the following combinations: 1 unit of insulin per kg, 1 unit of insulin per kg plus 2 g of dextrose for each unit of insulin, and 10 cc of water per kg. The insulin was administered subcutaneously, and the dextrose and water were given orally by stomach tube at the time the alcohol injections were completed. The water administrations were repeated hourly for 5 hours, making a total of 60 cc per kg for each animal.

Results. An inspection of Fig. 1 shows that all of the procedures tried actually interfered with the metabolic processes concerned with the removal of isopropyl alcohol from the blood stream. The greatest change in the slope of the blood alcohol curve was effected by insulin in combination with dextrose. The rate of decline of blood alcohol was only about one-half as fast with this combination as with alcohol alone. In the case of water diuresis and insulin, the rate of decline was three-fourths and five-sixths, respectively, that of the control. Acetone, an intermediate metabolite of isopropyl alcohol, showed a gradual increase in the blood from an average of 30 mg % to about 72 mg % from the first to the sixth hour. This fairly uniform rise in the 4 series of experiments indicated that the appearance of acetone in the blood was of no significance in detecting any acceleration in the rate of isopropyl alcohol metabolism. Urine acetone concentrations, except for a slight lag during the first hour, were about the same as those found in the blood. Isopropyl alcohol was excreted to some degree in the urine. Al-

* This report is part of a project which involves a complete pharmacologic investigation of isopropyl alcohol and is supported by the Standard Alcohol Company of New York, through the courtesy of Mr. James Park.

† Preliminary report appeared in *Fed. Proc.*, 1946, **5**, 189.

1 Lehman, A. J., Schwerma, H., and Rickards, E., *J. Pharm. Exp. Therap.*, 1945, **85**, 61.

2 Newman, H., and Abramson, M., *J. Pharm. Exp. Therap.*, 1942, **74**, 369.

3 Greenberg, L. A., and Lester, D., *J. Biol. Chem.*, 1944, **154**, 77.

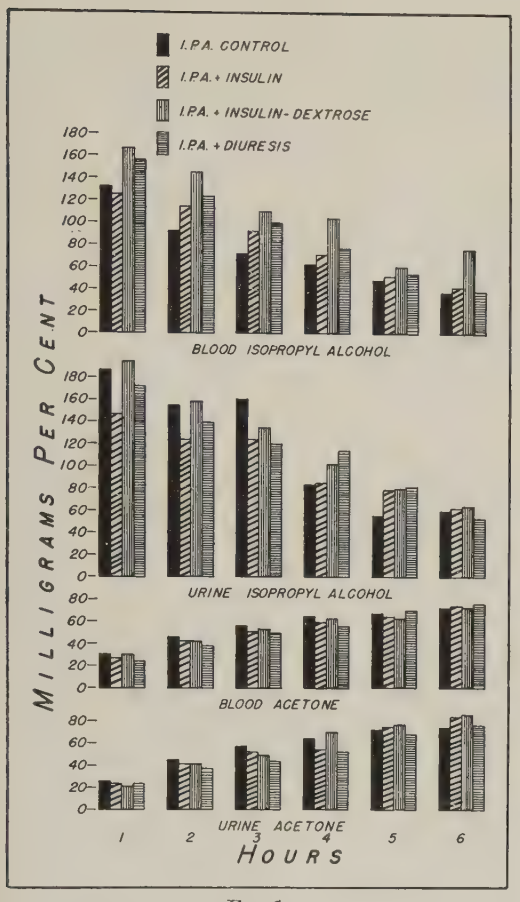

FIG. 1.

Blood and urine isopropyl alcohol and acetone concentrations after intravenous injection of 1.0 g per kilo of isopropyl alcohol (I.P.A.) in dogs, alone and in combination with insulin, insulin plus dextrose, and water diuresis. Each column represents the average of 3 observations.

though it is excreted more rapidly in diuresis, the blood alcohol concentrations remained higher than in the controls. It should be pointed out that the total excretion is a minor factor in regulating blood levels.[4] The effect of variations in the rate of excretion is obscured by the much larger variation in rate of metabolism. These results support the work of Pohl,[5] who tried a number of agents such as epinephrine, an extract of pituitary, histamine, etc., none of which appeared to have any effect on the rate of combustion of isopropyl alcohol.

Discussion. It is of interest to compare the urinary excretion of isopropyl alcohol with that of ethyl alcohol. This comparison shows some similarities as well as some differences. Like ethyl alcohol, the amount of isopropyl alcohol eliminated in the urine is not sufficient to influence the blood levels to any extent. From Fig. 1 it is evident that concentration of isopropyl alcohol in the urine does not occur. The alcohol is diffusible into body tissues.[6] After filtering at the glomerulus, it apparently diffuses freely out of the renal tubule as water is reabsorbed, so that the urine comes into concentration equilibrium with the blood. Consequently, it is not possible to hasten alcohol excretion except by increasing the volume of urine. In support of this, it may be pointed out that the control, insulin, and insulin-dextrose series of animals put out an average of 10 cc of urine per hour and excreted 0.49% of the total dose of isopropyl alcohol in 6 hours. The diuretic animals put out 128 cc of urine per hour and excreted 5.49% of the total dose of alcohol in 6 hours. Both urine flow and alcohol excretion increased with diuresis to about the same degree—in this case, approximately 12-fold.

Unlike ethyl alcohol, the concentration of blood isopropyl alcohol bears no fixed relationship to urine isopropyl alcohol. For ethyl alcohol, this is given as 1:1.35 and is independent of the volume of urine excreted. Calculations of this ratio for isopropyl alcohol gave values of 1:0.6 to 1:3.39 and were inconstant, regardless of the time of sojourn of the alcohol in the body or the amount of urine excreted.

No diuretic action attributable to isopropyl alcohol was noted in these experiments. Actually, an inhibition of urinary excretion was noted in the diuretic dogs. The fluid intake of each animal averaged 890 cc, and the volume of urine excreted averaged 769 cc, or a retention of 121 cc of water during the 6-hour period.

Summary. 1. Insulin in doses of 1 unit per kg, either with or without dextrose, and water diuresis were ineffective in influencing

4 Lehman, A. J., Schwerma, H., and Rickards, E., *J. Pharm. Exp. Therap.*, 1944, **82**, 196.

5 Pohl, J., *Biochem. Z.*, Berlin, 1922, **127**, 66.

6 Kemal, H., *Z. f. Physiol. Chem.*, 1937, **246**, 59.

the blood isopropyl alcohol curve in dogs given 1.0 g per kg intravenously. The predominant effect was a retardation of the rate of decline, and the use of these substances clinically in acute isopropyl alcoholic intoxication does not appear to be practical.

15431

Inhibitory Effect of Thiamine on Vasoconstrictor Action of Nicotine Tested in the Laewen-Trendelenburg Preparation.

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The inhibitory effects of sulfonamides, thiamine and thiazole compounds on the action of nicotine in isolated organs, such as small intestine and striated muscle have been reported previously.[1,2] Recently, investigations have been carried out to determine the effect of thiazole compounds on the blockade action of nicotine on the blood pressure of cats.[3] In view of the probable relationship between tobacco smoking and certain vascular diseases, especially thrombo-angiitis obliterans[4] on the one hand, and the peripheral vasoconstriction induced by nicotine on the other hand, an investigation on the effects of thiamine upon nicotinic vasoconstriction seemed to us of interest. The results reported here deal with such a study.

Methods. All the experiments were carried out using the Laewen-Trendelenburg (L-T) preparation in frogs (*Rana pipiens*). This method is highly sensitive for testing vasomotor substances. Male as well as female frogs were used. Frog Tyrode solution was used as a perfusate. Two Mariotte bottles were connected by a Y-piece with the perfusion system, *i.e.* the rubber tubing leading to the aortic cannula. One contained plain Tyrode solution, the other, dissolved in Tyrode, one of the following substances: crystalline thiamine hydrochloride (Merck), thiazole, pyrimidine, sodium sulfathiazole, thiouracil, para-aminobenzoic acid, nicotinamide. These substances were used at various concentrations. Nicotine in the form of nicotine base† or nicotine salicylate in aqueous solution was used. The injections of the different drugs were made through the rubber tubing into the glass cannula inserted into the abdominal aorta of the frog. All due precautions were used during the injection of drugs to avoid mechanical interference with the perfusion. The vasomotor action of the different drugs was measured in terms of the output variations of drops per minute.

Results. 1. *Control experiments with nicotine.* The injection of a small amount of nicotine (1-10 γ) in the L-T preparation elicited 2 major reactions: (a) Twitchings of the thigh and leg muscles, lasting as a rule from 30 to 60 seconds. (b) A marked reduction (50%-80%) of the outflow from the abdominal vein, as expressed in terms of drops per minute. The duration of the vasoconstrictor effect varied though the average ranged between 15 to 30 minutes until the output returned to its initial level.[5]

A series of experiments was carried out in which nicotine was tested several times in the same preparation. As a rule, the perfused frog became more sensitive to nicotine as the experiment progressed, *i.e.* a

* Fellows of the Dazian Foundation for Medical Research.

1 Pick, E. P., Brooks, G. W., and Unna, K., *J. Pharm. and Exp. Therap.*, 1944, **81**, 133.

2 Unna, K., and Pick, E. P., *J. Pharm. and Exp. Therap.*, 1944, **81**, 294.

3 Pick, E. P., and Unna, K., *J. Pharm. and Exp. Therap.*, in press, 1946.

4 Silbert, S., *J. A. M. A.*, 1945, **129**, 5.

† Nicotine Alkaloid Eastman Kodak.

5 Handovski, H., and Pick, E. P., *Arch. f. exp. Path. u. Pharmakol.*, 1913, **71**, 89.

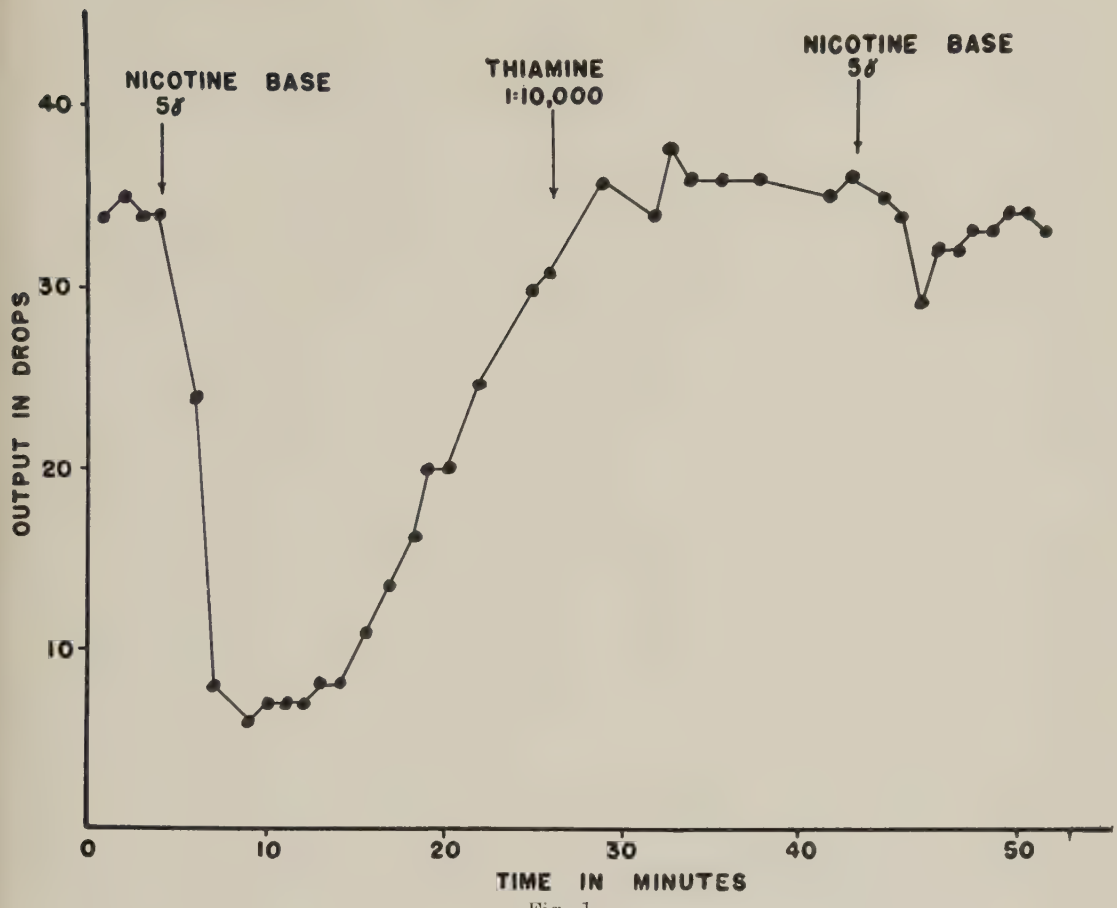

Fig. 1.
Inhibitory effect of thiamine (1:10000) on the vasoconstrictor action of nicotine.

small dose induced a more marked vasoconstriction. In some rare instances, however, a tolerance to nicotine took place. It was found that some frogs exhibit individual variations as to the doses of nicotine used, and some were entirely refractory to the usual small doses. In view of the above facts in the following series of experiments each individual preparation was first tested for its sensitivity to nicotine. Preparations exhibiting reduced reactivity to small amounts (1-10 γ) of nicotine were discarded. The best results obtained were those with nicotine base, which was used in most of the present work.

2. *Inhibitory effect of thiamine on nicotine.* The perfusion of the hind legs of the frog with a solution of thiamine hydrochloride in the concentration ranging from 1:10000 to 4:10000 completely inhibited the action of nicotine in most cases (Fig. 1). More dilute solution of thiamine was effective, although to a lesser degree, in preventing the nicotine vasoconstriction.

3. *Effective amount of thiamine retained in the tissues.* The perfusion with thiamine was usually allowed to run for 5 to 10 minutes before the tests were performed. Repeated nicotine injections showed that the vessels failed to constrict and the striated muscles failed to twitch. Since the L-T preparation is an open perfusion, it is self-evident that a large amount of the thiamine was passing only through the vessels and was being excreted. Only a partial amount would be retained therefore in the tissues. In order to evaluate the actual amount of thiamine fixed by the tissues we have

TABLE I.

Exp. No.	Thiamine input (γ) (10 min.)	Thiamine output (γ) (10 min.)	Amount retained (γ)	% retained
50	412	355	57	13.8
51	226	188	38	16.8

measured, in 2 perfusion experiments, fluorometrically, the input and output of thiamine over a period of 10 minutes. The difference thus evaluated (38-57 γ) represented the actual amount of thiamine fixed by the tissues. As can be seen from the following table (Table I) only from 13.8% to 16.8% of the perfused thiamine was retained and, of course, a very small part of it might be linked selectively on the specific nervous or muscle elements.

4. *Action of the individual moieties of the thiamine molecule on nicotine.* The 2 moieties of the thiamine molecule, 4-methyl-5-hydroxyethylthiazole and 2-methyl-5-ethoxymethyl-6-aminopyrimidine obtained in pure form‡ were tested individually. Thiazole in concentrations of 1 to 4 per 10000 cc of Tyrode solution, inhibited, partially or completely, the muscular twitchings and the vasoconstriction due to the action of nicotine (Fig. 2). The pyrimidine moiety, on the other hand, failed to counteract the effects of nicotine in the concentrations of 2 to 4 per 10000 cc of Tyrode.

5. *Action of related substances containing a thiazole or pyrimidine group on nicotine.* (a) In contrast to thiamine, sodium sulfathiazole antagonized the action of nicotine only in a relatively high concentration (4:1000), which was approximately 10 times as high as that of thiamine. Smaller doses (.1 to 2 per 1000) inhibited the nicotine action only slightly or not at all. It is noteworthy that in the proper concentration sulfathiazole exhibited not only an inhibitory effect on the vasomotor activity of nicotine but also abolished the muscular twitchings of the hind legs of the frog.

In connection with sulfathiazole, para-aminobenzoic acid was tested with no inhibitory effect. The same statement is true for nicotinamide.

(b) Thiouracil, which is a pyrimidine, used in concentrations of 1 to 4 per 10000 did not inhibit the action of nicotine.

Discussion. Nicotine used in the L-T preparation as test object, acts on both striated and smooth muscle. Thiamine, through its thiazole group, blocks these 2 actions. After excision of both sympathetic chains and resection of all spinal nerves of the hind legs, the injection of small amounts of nicotine (1-10 γ) still induces the same degree of twitching and vasoconstriction as before. This fact seems to indicate that the site of action of nicotine in the L-T preparation is not in the autonomic ganglia but more peripheral. It is well known that *striated muscle* can be stimulated by applying nicotine to it. "The twitchings are rarely, if ever, produced except from the region of the nerve endings." (Langley).[6]

As to the *smooth muscle* of the vascular bed, the site of action of *nicotine* seems to be either at the post-ganglionic nerve endings or at the neuro-effector cells. This would be in agreement with Stoehr's work on mammalian vessels[7] and with Krogh's work on the frog vessels,[8] according to the latter it is extremely improbable that local ganglion cells exist in the vessels of the extremities. On the other hand, since *thiamine* as shown previously[2] has an affinity for synapses, it is to be assumed that it is fixed at the myoneural junctions and thus blocks the action of nicotine.

Thiazole is the active group of the molecule, while pyrimidine is ineffective. These findings are in agreement with previous investigations on isolated smooth and striated muscles.[1,2] The intimate mechanism of the

‡ These substances were obtained through the courtesy of Dr. W. H. Engels of the Merck Research Laboratory, Rahway, N. J.

[6] Langley, T. N., *J. Physiol.*, 1907-1908, **36**, 347.

[7] Stoehr, P., Jr., *Mikrosk. Anat. d. veget. Nervensyst.*, Berlin, Springer, 1928, 67.

[8] Krogh, A., *The Anat. and Physiol. of Capillaries*, Yale University Press, 1924, 81.

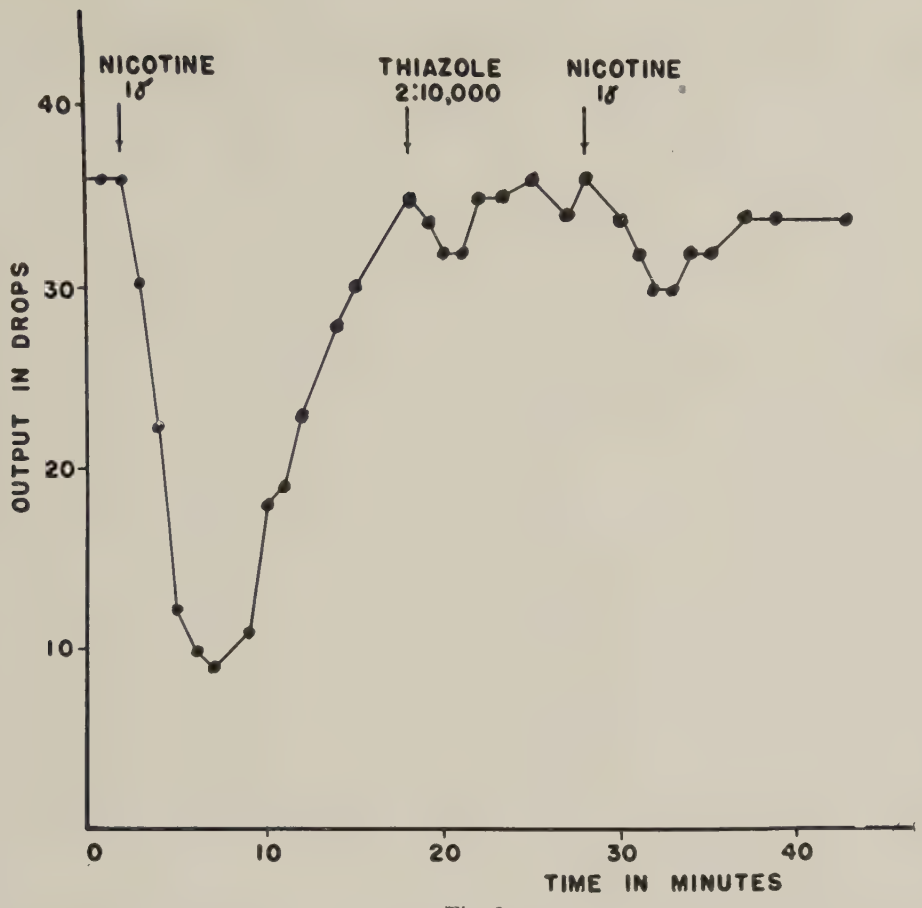

Fig. 2.
Inhibitory effect of thiazole (2:10000) on the vasoconstrictor action of nicotine.

antagonism between thiazole and nicotine was not investigated here.

Summary. 1. Thiamine, in very small concentrations (1:10000), inhibits the vasoconstrictor action of nicotine in the frog vessels. 2. The inhibiting action of thiamine is linked with the thiazole moiety of the molecule, the pyrimidine group being ineffective. 3. Thiouracil, nicotinamide, paraaminobenzoic acid do not inhibit the action of nicotine; sulfathiazole has an inhibiting effect only in a relatively high concentration (4:1000). 4. The site of action of thiamine seems to be at the myoneural junction in the striated muscles and at the postganglionic nerve endings or in the muscle elements of the vessel walls in the smooth muscles.

15432

Development of Immunity to Reinfection During Chemoprophylaxis of Trypanosomiasis with a New Antimony Derivative.

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The sodium salt derived from *p*-(2,4-diamino-1,3,5-triazinyl-6) aminophenylstibonic acid (Compound 122), exercises a long-lasting prophylactic activity in mouse-trypanosomiasis, according to Friedheim and Berman.[1] The duration of this protection varied with different animals, and in our own experiments we were able to protect mice treated with single doses of .05 g/kg for a period of up to 330 days. The mouse which was protected for the longest period finally succumbed from trypanosomiasis after it was previously inoculated 45 times without incident. Friedheim and Berman suggested that the prophylaxis against a first infection could be explained by a slow elimination of the drug. Indeed, at the site of injection a deposit of the free acid is formed which is resorbed very slowly. However, we also obtained a very long-lasting prophylactic effect when the product was given by mouth, in 25 and 50 mg/kg doses administered on 5 consecutive days; the mice were protected for a period of 2 months or more against 5 or less consecutive reinoculations. Further experiments are necessary before the mechanism of this prophylaxis can be determined.

During and at the end of the period of protection, certain phenomena occurred which indicate that immunologic processes had developed in the protected animals. First: In all control mice, the trypanosomes appeared in the blood stream 24 hours after the inoculation, whereas in all treated and protected mice, there was an interval of 4 to 8 days between the inoculation of trypanosomes and their appearance in the blood stream. Secondly: In several of the protected mice, trypanosomes appeared in the blood stream for a short time, as already pointed out by Friedheim and Berman. In other mice, they appeared as above, 4 to 8 days after a reinoculation, but did not multiply and disappeared spontaneously after a short interval.

The following experiments were made in order to determine whether these phenomena were due to a specific action of Compound 122, or to processes independent of the nature of the therapeutic agent.

1. Ten normal, uninfected mice received a subcutaneous injection of 50 mg/kg of Compound 122. Three weeks later their blood was collected and pooled and the serum obtained injected into normal mice. Twenty-four hours later these mice were inoculated with trypanosomes and succumbed, as usual, after 3 to 4 days. In a second series, 10 mice were inoculated with trypanosomes and 48 hours later, at the height of infection, treated with 50 mg/kg of Compound 122. Three weeks later the animals were bled, the blood pooled and the serum so obtained injected into untreated mice. These mice were protected against a subsequent infecting dose with trypanosomes.

Serum of normal mice served as a control and did not, as it has long been known, interfere with the infection. Repetition of these experiments under different conditions, especially varying the time between the curative treatment with Compound 122 and the collection of the serum, always gave the same results and showed that anti-trypanocidal effect was obtained 5 days after the treatment of the infected mice with Compound 122. The intensity of the protective and trypanocidal effect of the immune mouse serum was compared *in vitro* and *in vivo* with that of human serum and it was found that the immunologic power was almost the same with both sera.

[1] Friedheim, E. A. H., and Berman, Rose L., Proc. Soc. Exp. Biol. and Med., 1946, **62**, 131.

TABLE I.
Passive Immunity After Prophylaxis with Compound No. 122.

Mice treated with	Inoculation	Appearance of Trypanosomes. Days after injection of serum										
		0	1	2	3	4	5	6	7	8	9	10	11
1. Normal serum			+	++	+++	d							
			(+)	+	++	++++	d						
			++	++	+++	d							
2. Protected mice:			0	0	0	0	+	+++	d				
No. 122 + Trypanos.			0	0	0	0	++	++++	++++	d			
			0	0	0	0	0	0	0	0	+++	++++	d
			0	0	0	0	0	0	0	0	0	0	0
3. No. 122 alone			+	++	++++	d							
			(+)	+++	++++	++++	d						
4. Neoarsphenamine +			0	0	0	0	+	+++	++++	d			
Trypanosomes			0	0	0	0	++	++++	d				
			0	0	0	0	0	0	++++	++++	d		
5. Controls			(+)	+	++	+++	d						
			(+)	++	+++	d							

0 = No trypanosomes; (+) - ++++ = Different quantities of trypa nosomes in the blood; d = Dead.

TABLE II.

Passive Immunity After Immunization with Dead Trypanosomes.

Serum obtained days after active immunization, days	Delay of reinfection, days
0	0
3	0, 0, 3
5	3, 4, 5
10	2, 3, 3
14	5, 5, 5

These results indicate that the trypanocidal power of the serum of infected mice treated with Compound 122 is, apparently, not due to circulating antimony, but to other processes, probably immunologic in nature, and induced by the chemotherapeutic agent. But this action is not characteristic of the antimony compound used, since an experiment in which Neoarsphenamine was used instead of Compound 122 produced the same result. (Table I).

2. The development of anti-trypanocidal activity in the mouse-blood is probably an effect of any successful treatment of dourine with subsequent death of the protozoa. Immunization with killed trypanosomes, with results similar to those obtained in our experiments with Compound 122, has often been obtained, for instance, in mice infected with *Trypanosoma brucei* and treated with arsacetin.[2] We had the same results after immunization with *Trypanosoma equiperdum*. Fifteen mice were exsanguinated at the height of the infection, the blood citrated, pooled, and the contained trypanosomes killed by repeated freezing and thawing. This sterile blood, incapable of producing infection, was injected, 0.5 ml doses, into normal mice and groups of 3 inoculated with living trypanosomes immediately and after 3, 5, 10 and 15 days. The results are seen in Table II.

Conclusion. It is confirmed that a single intraperitoneal or subcutaneous injection of a sodium salt derived from melaminylphenylstibonic acid (Compound 122) confers a long-lasting protection against numerous consecutive infections of the mouse with *Trypanosoma equiperdum*. This protection is also obtained by oral administration of the compound. In infected mice which have been cured with this product, an immunity to reinfection with *Trypanosoma equiperdum* is achieved. The passive transfer of this immunity has been demonstrated. A similar state is reached if the product is injected first, and subsequently, reinfections with small amounts of trypanosomes are made. It is not established whether this acquired immunity to reinfection plays a role in the prophylaxis conferred by Compound 122.

[2] Browning, C. H., and Gulbranson, R., *J. Path. and Bact.*, 1936, **43**, 479.

15433

Pectin Adjuvant for Oral Penicillin.

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Numerous adjuvants have been used individually and in various combinations in attempts to increase the absorption of orally administered penicillin.[1-3] For the most part the relative efficacy of these substances has been determined by comparing the average blood concentrations obtained in small groups of subjects at various intervals following a dose or on the average amount of penicillin recovered from the urine. Individual variations under these conditions, how-

[1] Cutting, W. C., *et al.*, *J. A. M. A.*, 1945, **129**, 425.

[2] Free, A. H., Parker, R. F., and Biro, B. E., *Science*, 1945, **102**, 666.

[3] McDermott, W., *et al.*, *Science*, 1946, **103**, 359.

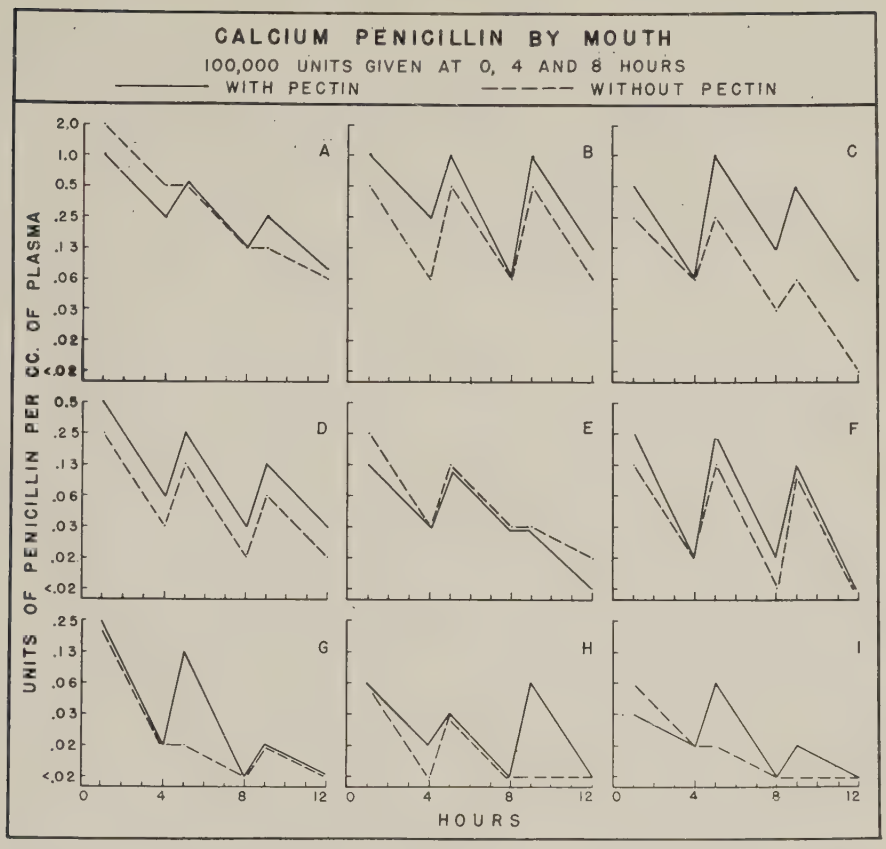

FIG. 1.

Penicillin levels in plasma after oral administration of calcium penicillin in gelatin capsules with and without a pectin adjuvant. Meals were given at 1¼, 5¼, and 9¼ hours.

ever, may be very great[3] and may profoundly affect the averages in small groups. For that reason it seems more appropriate to compare any given substances at different times in the same individuals under as nearly identical conditions as possible. A comparison of this sort was made with 2 oral preparations of calcium penicillin one of which contained a pectin derivative as an adjuvant.

Materials and Methods. The calcium penicillin used for the 2 preparations was predominantly penicillin G. In one of them the penicillin was conjugated with a pectin hydrolysate, pH 6.0-7.0, made from a high grade pectin which conformed to the standards prescribed therefor by the N.F. Both the control and the pectin preparation were contained in simple gelatin capsules without formalin, hardening or other preservatives added. Each capsule contained 25,000 units of penicillin.* The subjects were convalescent patients who had not recently received antibiotics or sulfonamide drugs. After an overnight fast, each subject was given a dose of 100,000 units of penicillin (4 capsules) at 6 a.m. and again at 10 a.m. and at 2 p.m. Blood for plasma penicillin levels was drawn 1 and 4 hours after each dose, the third and fifth samples being taken just before the second and third doses. Meals were served about 15 minutes after each of the one-hour bloods. Half of the subjects used each day received one of the preparations and the rest were given the other.

* These materials and the data concerning them were obtained through the kindness of Commercial Solvents Corporation.

TABLE I.

Average Plasma Penicillin Levels After Oral Administration of 2 Preparations of Calcium Penicillin* in the Same 9 Subjects.

Hour	Plasma level, units per cc	
	With Pectin	Without Pectin
1	.41	.42
4	.08	.08
5	.34	.18
8	.04	.03
9	.24	.11
12	.03	.02

* 100,000 units given at 0, 4, and 8 hours. Meals at 1¼, 5¼, and 9¼ hours.

Several days later the test was repeated in the same subjects, each receiving the alternate preparation. Plasma penicillin levels were done by a slight modification of the serial dilution method of Rammelkamp,[4] using the same strain of hemolytic streptococcus (No. 98).† The smallest amount of penicillin measured was 0.0156 unit per cc (shown as .02 unit in Fig. 1).

Results. The plasma penicillin levels obtained in 9 subjects are shown in Fig. 1. It is at once apparent that there are wide differences in the plasma levels obtained in individual subjects with either preparation. These differences are much greater than those obtained in the same subjects with the 2 substances. In addition, there was a general downward trend in the plasma levels of all of the subjects after the second and third doses. This may have been the cumulative influence of the meals.[5] While the differences between the plasma levels obtained with the 2 preparations in the same subjects were not striking, those obtained with the pectin-containing preparation were either the same or higher, and they were more often higher after the second and third doses. This is also evident from the higher average plasma levels obtained in the 9 subjects as shown in Table I.

Conclusion. Pectin hydrolysate used as an adjuvant for oral calcium penicillin resulted in somewhat better sustained plasma penicillin levels as compared with the same materials without the pectin.

[4] Rammelkamp, C. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 95.

† These determinations were done by Clare Wilcox.

[5] Finland, M., Meads, M., and Ory, E. M., *J. A. M. A.*, 1945, **129**, 315.

15434 P

Experiments on the Lateral Line System of Anurans.

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It has been claimed that in fishes[1,2] ordinary epithelial cells are transformed into lateral line sense organs under the influence of the regenerating lateral line nerve. However, in 19 *Rana clamitans* tadpoles observed frequently under the compound microscope for as long as 200 days, no new sense organs developed in association with the regenerating lateral line nerve fibers growing under skin not originally containing these organs.

In another series of experiments living lateral line sense organs were observed during the regeneration of tail tips following tail-tip amputation in 53 Rana tadpoles. Thirty-two were hypophysectomized animals, which, due to the reduction of skin pigmentation, are of great value in making observations upon the sense organs, peripheral nerves and blood vessels. Twenty-one were normal tadpoles. In 17 control cases the lateral line nerve supplying the regenerating

[1] Brockelbank, M. C., *J. Exp. Zool.*, 1925, **42**, 293.

[2] Bailey, S. W., *J. Exp. Zool.*, 1937, **76**, 187.

series of organs was allowed to regenerate normally. In the 36 experimental cases the lateral line nerve was transected anterior to the level of tail-tip amputation and was resectioned sufficiently often to prevent reinnervation of the posterior portion of the stump and the regenerating series of organs. In 10 of these the lateral line nerve was severed after regeneration of sense organs had begun; in 19, it was severed at the same time as tail-tip amputation; and in 7, it was cut 7-10 days previous to tail-tip amputation. In these 3 experimental series, as well as in the controls, placodal material developed from the most posterior organ on the stump or from one of the organs of the most posterior cluster. These cells rapidly proliferated and usually within 10 days formed a long chain of interconnected clumps of cells extending posteriorly on the regenerating tail tip. No contributing placodal material ever developed from the more anterior organs. The clumps of cells, possessing at first no hair cells, differentiated into typical, though small, lateral line-sense organs with sensory cells possessing hairs. The organs eventually attained the same size as those removed by amputation. The progress of differentiation was directed posteriorly, *i.e.*, the regenerate organs which were first fully differentiated were those nearest the original site of amputation, and a wave of differentiation proceeded posteriorly. There never was the slightest indication that denervation before, after, or simultaneously with tail-tip amputation had any effect upon the time or mode of regeneration of lateral line sense organs,* and it is, therefore, concluded that any trophic or inductive influence on the part of the lateral line nerve is unnecessary for normal regeneration of the sense organs.

Further evidence that there is considerable independence of the sense organs of their nervous connections is demonstrated by a third type of experiment. Living, denervated, lateral line sense organs of the dorsal tail fin were observed and compared with normally innervated organs on the opposite side over extended periods of time. Sixty-seven experiments, involving repeated observations on a total of 2,313 lateral line sense organs were followed for 6-142 days. These included 37 experiments on hypophysectomized Rana species and 30 on normal *Rana clamitans* tadpoles. The normally occurring degeneration of lateral line sense organs and nerves due to metamorphosis is circumvented by hypophysectomy. In no instance was there any immediate apparent effect of denervation on the sense organs, but only after prolonged denervation was the removal of the trophic action of the nerve on the sense organs made visible by the degeneration of some sense organs.* However, there was great variation in the ability of these organs to withstand degeneration. Some still appeared normal after 142 days of denervation, while others degenerated as early as 39 days after denervation. Degeneration of the denervated organs could not be correlated with any lack of proper blood circulation and must be attributed rather to the lack of specific nervous connections. A far greater dependence of the lateral line sense organs on the trophic influence of the lateral line nerve of some of the fishes has been reported.[1,2]

In 2 cases where some lateral line organs had degenerated the nerve was allowed to regenerate. There was no reappearance of organs in the position of the degenerated organs. Again, this indicates an apparent lack of inductive influence of the lateral line nerve in the formation of sense organs.

* This is in agreement with the observations reported in abstract by Speidel, C. C., *Anat. Rec.*, 1944, **68**, 458.

15435

Infectivity of *Hemophilus pertussis* for the Chick Embryo.

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For a long time the study of *Hemophilus pertussis* infections in the laboratory was hampered by the lack of a suitable animal of high susceptibility. White mice have been used frequently as the experimental animal, but in this species fatal infections are not regularly obtained through the usual routes of inoculation unless very large numbers of *H. pertussis* are administered. Recently Kendrick[1] has found that by the intracerebral route a much smaller dose of organisms will suffice to initiate fatal infection and this observation has been confirmed by other workers in studies on the chemotherapeutic activity of streptomycin[2] and the antigenic potency of vaccines derived from fluid cultures;[3] the LD_{50} in their experiments appeared to be a few hundred organisms.

In the course of studies which revealed the susceptibility of the developing chick embryo to a large variety of viruses and bacteria, Gallavan and Goodpasture[4] showed that *H. pertussis* would produce infection in 11- to 13-day embryos following introduction via the chorio-allantois or amnion. They did not attempt to study the quantitative aspects of the infection but did note that animals surviving 6 days or longer occasionally yielded positive heart's blood cultures. With this in mind, we thought it might be worthwhile to examine the susceptibility of the chick embryo to infection via the yolk-sac or allantoic cavity since these routes have proved so valuable in the investigation of other pathogenic agents.

Materials and Methods. The experiments were carried out with *H. pertussis* strain 934 obtained through the kindness of Dr. P. Kendrick of the Michigan State Department of Health. Cultures were propagated on sheep blood potato-infusion agar plates; typical hemolysis and other phase I characteristics were maintained throughout. After incubation for 48 or 72 hours at 37°C, the growth was removed and serial dilutions prepared in N-Z casein digest broth. To determine the number of viable organisms in the inoculum 0.1 ml of suitably diluted suspension was distributed over the surface of plates of the Bordet-Gengou medium with the aid of a glass spreader. Colony counts were made after 72 or 96 hours' incubation at 37°C.

Incubated fertile eggs were obtained from a local hatchery; no effort was made to secure eggs from a single breed of hen. For the yolk-sac route 0.5 ml of a given dilution was employed as inoculum; 0.1 ml was injected in the allantoic cavity. The eggs were subsequently incubated at 34-35°C and candled daily to determine the viability of the embryo. When the eggs were opened, cultures and smears stained by Wright's method were made from the yolk and allantoic fluid. Cultures of amniotic fluid, tracheal fluid, and heart's blood were also made from time to time.

Experimental Data. When fertile eggs of 7 to 11 days' preliminary incubation were infected via the yolk-sac with a small number (20 or less by plate count) of viable *H. pertussis,* death of the embryo usually resulted 6 to 9 days after inoculation. At autopsy, smears and cultures of the yolk showed numerous organisms. Occasionally small numbers of *H. pertussis* were recovered

[1] Kendrick, P., unpublished data, cited by Hegarty *et al.*[2]

[2] Hegarty, C. P., Thiele, E., and Verwey, W. F., *J. Bact.,* 1945, **50**, 651.

[3] Cohen, S. M., and Wheeler, M. W., *Am. J. Pub. Health,* 1946, **36**, 371.

[4] Gallavan, M., and Goodpasture, E. W., *Am. J. Path.,* 1937, **13**, 927.

TABLE I.
Infectivity of *H. pertussis* via the Yolk Sac in the Chick Embryo.

Inoculum				
Source	Dilution	No. of viable *H. pertussis*	Age of eggs when infected (days)	Results*
B-G culture	10^{-7}	144	7	D6, D7, D8
			11	D7, D9, 0
	10^{-8}	14	7	D6, D8, 0
			11	D6, D7, 0
	10^{-9}	1.4	7	D8, 0, 0
			11	D8, 0, 0
Infected yolk	10^{-8}	15	8	D5, D8, D9, D9
	$1/3 \times 10^{-8}$	5	8	D6, D7, D8, D8, D9
	$1/9 \times 10^{-8}$	1.7	8	D5, D6, D6, S10, 0
Infected allantoic fluid	10^{-9}	2	8	D8, D9, D9
	$1/3 \times 10^{-9}$	0.7	8	0
			12	0, 0

* Results:
D_6 signifies death after 6 days' infection with many *H. pertussis* in yolk.
0 signifies no evidence of infection.
S_{10} signifies survival for 10 days, with positive culture from yolk.

in cultures from the heart's blood and amniotic fluid; none were found in the allantoic fluid. The heavily-infected yolk of embryos succumbing to infection was diluted in broth and titrated by the same route in other embryos. As few as 5 organisms deriving from the yolk were found to be capable of initiating a fatal infection. Table I summarizes experiments which exemplify these results.

H. pertussis from cultures on Bordet-Gengou medium or from heavily-infected yolk were also introduced via the allantoic cavity of 10- or 11-day embryos. A considerably larger number of organisms, usually at least several thousand, was necessary for the initiation of a fatal infection and deaths were infrequent. In those embryos succumbing, however, many organisms were seen in smears of the allantoic fluid and cultures revealed them to be present also in the heart's blood and tracheal fluid. *H. pertussis* in the allantoic fluids proved to be of high virulence when tested by the yolk-sac route (Table I).

Conclusion. The chick embryo is highly susceptible to *H. pertussis* when infected via the yolk-sac. The results of inoculation via the allantoic cavity are much less satisfactory. The chick embryo would seem to promise considerable utility in the quantitative experimental evaluation of antibody preparations and chemotherapeutic compounds versus *H. pertussis*.

We are grateful to Mrs. I. B. Heilbrunn for assistance during the early phase of this work.

15436

Prothrombin Level and Effect of Vitamin K Substitutes in Thrombocytopenic Purpura in Rats.*

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The administration of antiplatelet serum to animals produces purpura, a prolonged bleeding time and a low capillary resistance. These abnormalities are generally attributed

* Aided by a grant from the Baruch Committee on Physical Medicine to Columbia University.

to the low level of blood platelets and a vascular defect.[1] An additional etiological factor has been suggested by the recent results of Fleck and Lille,[2] who reported that the blood prothrombin level is reduced in rats and guinea pigs which had been given antiplatelet serum. According to these workers, the "symptoms" of experimental purpura can be almost completely inhibited by the prophylactic administration of the vitamin K substitute 2-me-1,4-naphthohydroquinone acetate. Their abstract does not indicate whether or not the vitamin prevents the fall in the prothrombin level or platelet count. Others in their laboratory have reported[3] that in human essential thrombocytopenia (Werlhof's disease), in which the prothrombin time is unchanged, the vitamin K substitute "stops the hemorrhagic symptoms and restores the bleeding time to normal" without raising the platelet count. Since these results, if correct, would necessitate a radical revision of our concepts of the action of vitamin K and of the pathological physiology of thrombocytopenic purpura, it seemed important to test their validity. Consequently, the experiments of Fleck and Lille on experimental purpura were repeated.

Methods. Purpura was produced in rats by the subcutaneous injection of antiplatelet serum. The serum was prepared by the intravenous injection of a rabbit with a saline suspension of the washed blood platelets of 10 or 11 heparinized rats. Four injections were given at approximately weekly intervals. Five days after the last injection, the rabbit was bled and the serum was preserved in a frozen state until within 10 days of its use.

Four pairs of white rats (Sherman strain, 210 to 330 g) were used in each of 3 experiments. Each pair consisted of a male and a female. Pair 1 received no injections, pair 2 received vitamin K, pair 3 was given vitamin K and antiplatelet serum, and pair 4 received only antiplatelet serum. The platelet count, prothrombin time and capillary resistance were determined. Blood for the platelet count was obtained by cutting the tail, and was diluted with 3.8% sodium citrate. Two methods were used to measure the prothrombin time. It was usually determined by Quick's micromethod,[4] in which about 10 mm^3 of whole blood was taken from the tail and added to an equal amount of thromboplastin solution.† At the end of each experiment, the prothrombin time was determined on oxalated plasma[5] which was obtained from cardiac blood samples (nembutal anesthesia). The capillary resistance was measured on the abdominal skin by Dalldorf's method.[6]

The 3 experiments differed in the dosages of antiplatelet serum and vitamin K, and in the vitamin preparation employed. Control determinations were made on all rats on the first day. In the first experiment, pairs 2 and 3 were given 10 mg/kg of 2-me-1,4-naphthoquinone bisulfite‡ intramuscularly on the first day. Antiplatelet serum (0.75 cc/kg) was administered subcutaneously to pairs 3 and 4. Determinations were repeated on the second and third days. On the fourth day, the rats were autopsied after taking cardiac blood samples. In the second experiment, 5 mg/kg of 2-me-1,4-naphthohydroquinone diphosphoric ester tetra sodium salt§ were injected into pairs 2 and 3 on the first day. The injections were repeated twice daily on the second and third days. Pairs 3 and 4 received 0.96 cc/kg of antiplatelet serum on the second day. Measurements were repeated on the third and fourth days. In the third experiment, 40 mg/kg of 2-me-1,4-naphthoquinone bisulfite‖ was administered to pairs 2 and 3 on the first, second and third days. Since the volume of fluid injected was large, the remaining rats received 10 cc/kg of isotonic saline as a control measure. Anti-

[1] Elliott, R. H. E., Jr., and Whipple, M. A., *J. Lab. and Clin. Med.*, 1940, **26**, 489.

[2] Fleck, L., and Lille, F., *Am. Rev. Soviet Med.*, 1945, **3**, 174.

[3] Groër, F., Baranowski, T., and Rosenbusch, J., *Am. Rev. Soviet Med.*, 1945, **3**, 173.

[4] Quick, A. J., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 788.

† Bacto thromboplastin from rabbit brain, Difco Laboratories.

[5] Quick, A. J., *Am. J. Clin. Path.*, 1940, **10**, 222.

[6] Dalldorf, G., *J. Exp. Med.*, 1931, **53**, 289.

‡ 2.5 cc/kg Hykinone, Abbott Laboratories.

§ 1 cc/kg Synkayvite, Hoffmann-La Roche, Inc.

‖ 10 cc/kg Hykinone.

TABLE I.
Effect of the Administration of Antiplatelet Serum to Rats 3 and 4, and of Vitamin K to Rats 2 and 3.

		1st day			2nd day			3rd day			4th day
Exp.	No., sex	Platelet count, per mm³ × 1000	Prothr. time, sec.	Cap. res.,* cm Hg.	Platelet count, per mm³ × 1000	Prothr. time, sec.	Cap. res.,* cm Hg.	Platelet count per mm³ × 1000	Prothr. time, sec	Cap. res., cm Hg	Plasma prothr. time, sec
1	1 ♂	600	40	30	866	49	20	940	45	19	20.5
	1 ♀	914	41	26	898	44	30	722	46.5	>25	19.4
	2 ♂	808	45	30	1218	51	20	766	40	25	19.9
	2 ♀	682	46	20	958	49	25	1042	39	24	23.4
	3 ♂	976	44	30	294	49	10	74	42	5	23.6
	3 ♀	777	36	28	176	37	15	16	40	10	22.2
	4 ♂	856	40	>30	350	36	20	20	34.5	15	21.4
	4 ♀	584	43	24	194	48	10	46	34.5	10	19.5
					3rd day			4th day			
2	1 ♂	640	30	17	874	36	26	818	39	>25	21.4
	1 ♀	642	30	>25	606	36	>26	820	33	20	20.8
	2 ♂	616	35	15	868	36	>26	834	39	>25	20.3
	2 ♀	812	37	18	818	37	27	770	38	>25	20.1
	3 ♂	928	30	18	254	35	20*	150	40	10	19.4
	3 ♀	681	31	>23	62	35	>27*	—	—	—	—†
	4 ♂	910	30	22	220	32.5	>26*	78	30	20	20.2
	4 ♀	724	35	>24	72	34	>24*	214	35	15	19.8
3	1 ♂	1346	42	35	662	39	25	480	—	10	24.6
	1 ♀	1202	35	15	1130	40	25	1190	—	15	21.6
	2 ♂	1156	40	30	224	40	25	700	—	>26	21.0
	2 ♀	934	36	15	1136	42	35	1012	—	27	21.0
	3 ♂	612	32	35	210	—	30	64	—	20	22.0
	3 ♀	802	40	35	136	32	20	168	—	10	21.0
	4 ♂	880	32	30	64	44	10	164	—	>25	20.4
	4 ♀	942	40	25	56	38	20	140	—	15	19.5

* These high values can probably be attributed to cutaneous vasoconstriction following excessive bleeding from the tail. The following determinations of capillary resistance, except those done on the first day of experiment 3, were therefore made before the animals were bled.

† This rat died during the night, probably as a result of blood loss.

platelet serum (0.75 cc/kg) was given to pairs 3 and 4 on the second day, and determinations were made on the third and fourth days.

Results and discussion. All of the rats which received antiplatelet serum (pairs 3 and 4) developed low platelet counts, and most of them showed a diminished capillary resistance (Table I). These changes were as great in the rats which received vitamin K (pair 3) as in the untreated rats (pair 4). Autopsies failed to reveal any difference in the degree of purpura in the skin and internal organs of the 2 groups. The purpuric rats usually bled profusely from the tail incisions whether or not the animals had been given vitamin K. The prothrombin time was unchanged in all experiments. Large doses of vitamin K lower the prothrombin time when diluted plasma is used in this test.[7] Our failure to observe a similar reduction may be attributed to the use of undiluted plasma, a less sensitive indicator of changes in prothrombin level.

Since Fleck and Lille gave no specific data concerning their criteria of therapy or the dosage schedule of vitamin K, an exact duplication of their work could not be carried out.¶ In the experiments reported here, very large doses of 2 vitamin K substitutes were used, and these were administered both before and after the injection of antiplatelet serum. The

[7] Field, J. B., and Link, K. P., *J. Biol. Chem.*, 1944, **156**, 739.

¶ Abstracts, written in English, were sent to the *American Review of Soviet Medicine* by the authors in Lvov. A more detailed account of the work is not available.

vitamin K substitutes which we employed were not identical with that used by the Polish workers. Since their compound and the 2 used in this study possess marked vitamin K activity,[8,9] it is most unlikely that our failure to confirm their work can be attributed to this difference. We can offer no explanation for the divergent results.

Summary. Contrary to the results of Fleck and Lille, the prothrombin level is not reduced after the administration of antiplatelet serum to rats, nor are the manifestations of experimental thrombocytopenic purpura modified by large doses of vitamin K substitutes.

8 Dam, H., *Advances in Enzymology*, 1941, **2**, 285.

9 Smith, J. J., Ivy, A. C., and Foster, R. H. K., *J. Lab. and Clin. Med.*, 1943, **28**, 1667.

15437 P

Control of Salmonella Infections in Mice by Streptomycin.

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Preliminary observations on the use of streptomycin to control paratyphoid infections in mice indicated that this antibiotic given orally may be an effective means of eliminating Salmonella infections in colonies of mice and other laboratory animals for considerable periods of time. This paper reports the results of work* started in November 1945 when it was found that Salmonella organisms were responsible for both decreased production of mice needed for the Japanese B encephalitis vaccine program and also were the cause of contamination of the final vaccinal product.

A commercial mouse colony which was supplying mice for the Japanese B program was selected for streptomycin feeding since mouse brain vaccine prepared from these mice contained Salmonella organisms. Individual fecal samples were collected from the 1400 breeders in the colony, and streaked on brilliant green agar. Of this number, 30 adult mice in 6 breeding units yielded positive cultures (*Salmonella enteritidis*). Streptomycin† was incorporated in the drinking water of the entire colony for 7 consecutive days so that the daily intake per mouse was approximately 100 units. Two weeks later 100 young mice were used from the same 6 Salmonella positive units for vaccine production. No paratyphoid organisms were found in the vaccine produced from this group. The 30 positive adult mice and 24 of their offspring were tested 4 weeks after the streptomycin was fed and at monthly intervals thereafter. Only one adult mouse yielded a positive fecal culture in the first test, and all 4 subsequent tests on both young F_1 and old mice were negative for the presence of paratyphoid organisms.

As a further development of these findings experiments are now in progress to study the effect of 25, 50, 100, 200, 400 and 1000 units of streptomycin daily on the spread of *S. enteritidis* infection in groups of mice consisting of infected and non-infected individuals. The results to date with one strain of *S. enteritidis* are encouraging but incomplete. In addition, mice have been injected with *S. enteritidis* and simultaneously with *S. typhi-murium* cultures isolated from mice of 20 other commercial colonies. In mice receiving the combined injection it was found that *S. enteritidis* was more susceptible to streptomycin than *S. typhi-murium*.

* This work was conducted under the Rockland Farms Research Fund.

† We are greatly indebted to Dr. Gladys Hobby of Chas. Pfizer & Co., New York, for the streptomycin used in these studies.

15438

The Components of Prothrombin.

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The author[1] presented evidence that prothrombin appears to be composed of calcium and 2 components which he designated as A and B. It was postulated that the 2 components were linked through calcium and that removal of the latter element caused the complex to split with the liberation of the 2 components. Thus, component A in undecalcified or native plasma disappears very slowly when stored, in contrast to its instability in oxalated plasma. Furthermore, component B which is promptly removed with aluminum hydroxide from oxalated plasma, is not effectively adsorbed by this agent in heparinized plasma which has not been decalcified.

In this study the stability of component A in native (undecalcified) hemophilic plasma was demonstrated. (Table I). The keeping quality of component A does not appear, however, to be as good in human as in avian blood.

TABLE I.
Effect of Storage* on the Prothrombin Time of Native and Oxalated Hemophilic Plasma.

Age of plasma, hrs	Prothrombin time in seconds	
	Native plasma†	Oxalated plasma
0	11½	11½
24	12½	15
48	12½	18

* The centrifuged blood was kept in celluloid test tubes and stored in a refrigerator.

† The prothrombin time was determined by adding 0.1 cc saline solution and 0.1 cc thromboplastin to 0.1 cc of native plasma.

Since native hemophilic plasma is not coagulated when aluminum hydroxide is added, as occurs in normal plasma, the adsorption of component B by this agent could be studied without the use of heparin, which was used in the original investigation. On mixing 2 cc of hemophilic plasma (without the removal of the calcium) with 0.1 cc of aluminum hydroxide cream, and incubating for 10 minutes at 37°C, component B was completely removed as indicated by the fact that on treating 0.1 cc of this plasma with 0.1 cc of a highly potent thromboplastin, no coagulation occurred. It may be concluded that component B can be adsorbed by aluminum hydroxide from native plasma, and that the inability of aluminum hydroxide to absorb it from heparinized plasma is apparently caused by an alteration of component B by heparin and is not due to a firm union of the component in the prothrombin complex as the writer formerly believed.

In order to test whether the fibrinogen is affected in stored plasma as Lavergne and Lavergne-Poindessault[2] have claimed, the following experiment was carried out:

Three cc of oxalated rabbit plasma was mixed with 0.2 cc of aluminum hydroxide cream, incubated at 37°C for 10 minutes, and then centrifuged. The clear plasma was treated with 0.01 cc of thrombin prepared according to the author's method.[3] After 10 minutes the fibrin was removed. The complete removal of the fibrinogen was shown by the fact that on mixing 0.1 cc of the plasma with 0.1 cc of thrombin, no further coagulation occurred. To permit neutralization of the small amount of added thrombin by the natural antithrombin, the plasma was allowed to stand 30 minutes. This plasma is designated as defibrinated alumina plasma. It contains neither fibrinogen nor component B.

Human oxalated plasma was stored in a

1 Quick, A. J., *Am. J. Physiol.*, 1943, **140**, 212.

2 Lavergne, G. H., and Lavergne-Poindessault, B., *C. R. Soc. de biol.*, 1942, **136**, 445.

3 Quick, A. J., *Am. J. Physiol.*, 1936, **115**, 317.

refrigerator for 7 days. During that period the prothrombin time increased from 12 to 28 seconds. On adding 1 part of defibrinated alumina plasma to 3 parts of this stored human plasma, a mixture was obtained which had a prothrombin time of 10 seconds. Since the only reacting fibrinogen was supplied by the stored plasma, the striking decrease of the prothrombin time brought about by the addition of alumina plasma to stored plasma must have been due to a specific factor which is not related to fibrinogen and which for the sake of simplicity has been named component A.

In this study the procedures which were employed have been previously described.[1]

Summary. 1. Undecalcified hemophilic plasma shows markedly less loss of prothrombin activity on storage than does oxalated plasma. 2. Aluminum hydroxide removes component B from native hemophilic plasma. 3. The prolonged prothrombin time in stored human plasma is strikingly shortened by the addition of defibrinated plasma treated with aluminum hydroxide. This proves that the delayed prothrombin time in preserved plasma is not due to an alteration of the fibrinogen.

15439

Effects of Injections of Equine Gonadotrophin upon the Gonads and Adrenals of Fetal Rats.*

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Using a method recently described,[1] fetuses of the Sprague-Dawley strain of rats were transferred to the abdominal cavity of the mother and subjected to subcutaneous injections of equine gonadotrophin. Thirty-two fetuses received injections twice daily and about 12 hours apart. Near term, the 16 that had survived were secured by severing the umbilical cord. Fourteen and certain littermate controls were immediately autopsied, while 2 were placed in the nest of a foster mother. Although one was promptly destroyed by the mother, the other one received 3 additional injections before it was sacrificed by decapitation (Fetus 20, Table I). Litter 112 (Fetuses 35 and 36) differs from others in that the mother received subcutaneous injections of progesterone for the purpose of delaying parturition. Injections, each containing 1.0 mg, were made twice daily on days 20, 21 and 22 of pregnancy. In all cases, the procedure at autopsy was to open the abdominal cavity before placing the specimen in Bouin's fixing fluid. After fixation, the lumbar and pelvic regions of the body were sectioned serially and the sections stained with hematoxylin and eosin. In some instances, certain sections of the series were segregated for special staining (Dominici's, Mallory's triple and Heidenhain's iron hematoxylin).

When sections of testes were examined with the aid of a micro-comparator, an instrument which brings half the field of each of 2 microscopes into view, it was found that the interstitial cells of Leydig of each injected male were larger and more abundant than those of litter-mate controls (Fig. 1 to 4). They were most abundant in the 2 fetuses which received the most hormone (3 and 5, Table I).

Although it is reasonable to suppose that the interstitial cells secreted androgen more rapidly than normally (?), evidence that they actually did could not be detected by inspecting serial sections of such developing

* This investigation has been aided by a grant from the medical research funds of the Graduate School. The writer is indebted to Dr. Dwight Ingle of The Upjohn Company for supplying the gonadotrophin (Gonadogen) and to Dr. Ernst Oppenheimer of Ciba Pharmaceutical Products, Inc., for furnishing the progesterone (Lutocylin).

1 Wells, L. J., *Anat. Rec.*, 1946, **94**, 530.

TABLE I.
Effects of Gonadotrophin upon Organs of Fetuses.

Fetuses	Litters	Treatment: Days of preg.	Treatment: No. of injections	Treatment: Total gonadogen (I U.)	Autopsy (hrs before term)*	Gonad: Vol. (cu mm)	Gonad: Tubules: Diam. (μ)	Gonad: Tubules: No. in matched section†	Adrenal (vol., cu mm)
					Males				
1	132		0		10	1.031	45.9	116	.439
2	”		0		10	1.014	43.3	108	
3	”	20-21	4	1186	10	1.065	51.7	81	.433
4	129		0		11	.865	48.8	90	.356
5	”	20-21	4	1032	11	.999	51.7	71	.353
6	137		0		1	1.212	50.7	89	.321
7	”		0		1	.971	46.5	108	
8	”	20-22	5	400	1	1.237	50.7	77	.292
9	136		0		1	2.064	50.5	87	.431
10	”		0		1	1.234	45.9	99	
11	”		0		1	1.096	45.9	110	
12	”	20-22	5	400	1	1.456	50.4	78	.329
13	”	20-22	5	400	1	1.280	50.1	56	.360
14	139		0		1	1.057	48.1	96	.372
15	”		0		1	1.052	49.8	112	
16	”	20-22	5	240	1	1.429	51.4	72	.363
17	”	20-22	5	240	1	1.175	48.8	97	
18‡	103		0			1.890	48.8	108	
19‡	117		0			1.735	48.5	120	
20§	119	20-21	7	800		1.126	45.3	90	
					Females				
21	130		0		10	.094			.271
22	”		0		10	.078			
23	”	20-21	4	1186	10	.090			.353
24	”	20-21	4	1186	10	.089			
25	132		0		10	.085			.388
26	”	20-21	4	1186	10	‖			‖
27	129		0		11	.084			.302
28	”	20-21	4	1032	11	.092			.313
29	114		0		9	.076			.378
30	”	20-22	5	500	9	.088			.411
31	”	20-22	5	500	9	.073			
32	115		0		9	.095			.367
33	”		5	500	9	.101			.502
34	”		5	500	9	.086			
35¶	112		0			.105			.369
36¶	”	20-22	6	600		.100			.339

* Hour of expected parturition estimated by allowing 21 days and 16 hours after observation of coitus.

† See text for method of matching sections.

‡ Control, autopsied at 36th hour after normal parturition.

§ Received 4 injections before severance of cord (9 hours before term) and 3 injections while in nest of foster mother and then was autopsied during 48th hour after severance.

‖ Loss of several sections of series militated against determining volume.

¶ Umbilical cord severed during 9th hour following normal term, mother having received progesterone (see text).

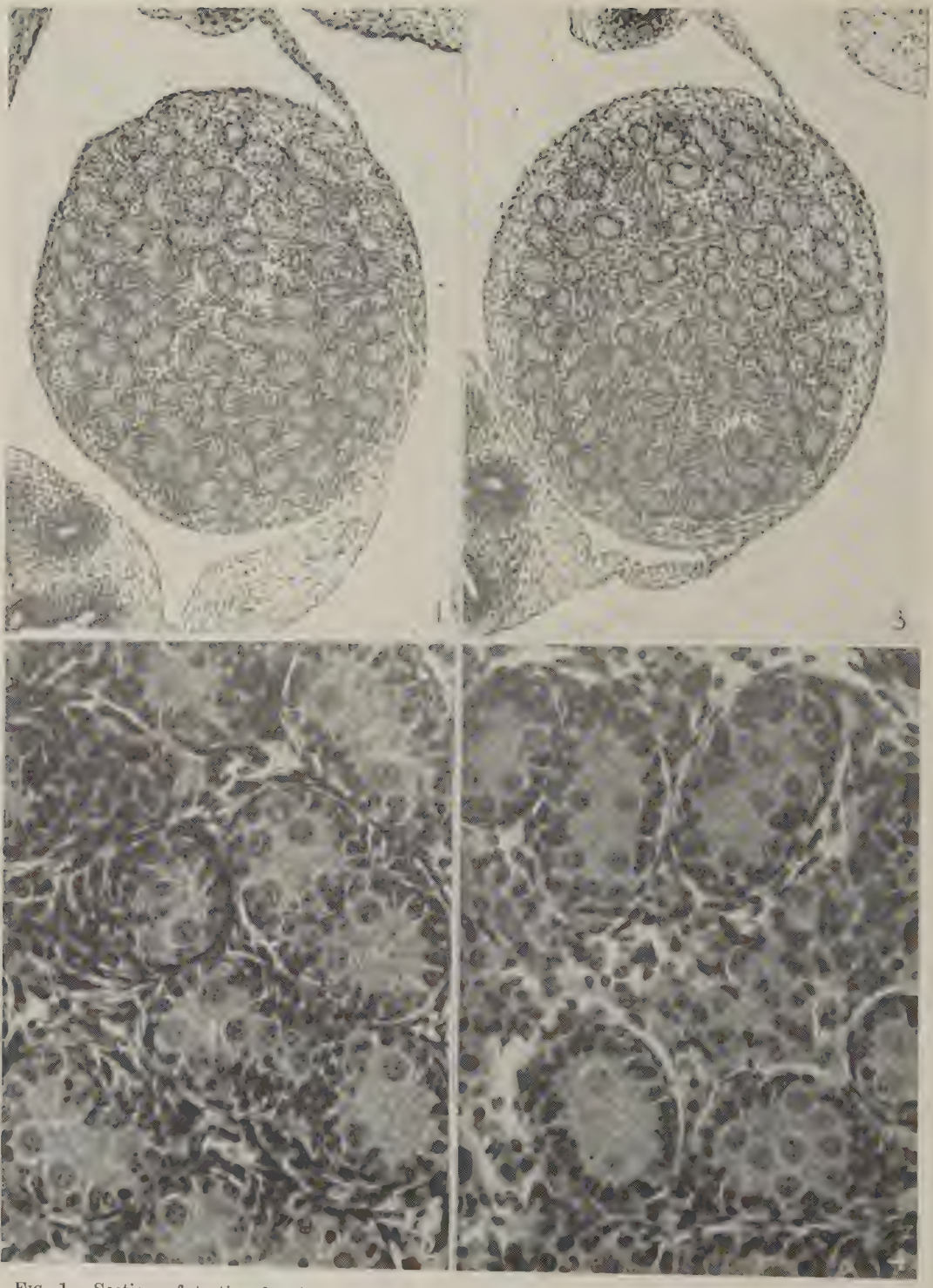

FIG. 1. Section of testis of uninjected control (Fetus 4). × 70.

FIG. 2. Higher magnification (Fetus 4). × 375.

FIG. 3. Section of testis of littermate which received 1032 I.U. of gonadotrophin (Fetus 5). × 70.

FIG. 4. Higher magnification (Fetus 5). × 375.

organs as the prostate, the seminal vesicles and the bulbo-urethral glands. Price and Ortiz[2] report that in rats injected after parturition and autopsied on the sixth postnatal day, the combined weight of the seminal vesicles and the coagulating glands was 25% greater than in controls.

The volume of the testis was determined by the paper-weight method of Hammar,[3] using the formula presented by Boyden,[4] with the modification that every tenth section was drawn and the combined weights were multiplied by 10. From these data alone it would seem that the gonadotrophin caused little or no growth of the testis.

In a study of the seminiferous tubules it was found that the gonadotrophin caused no significant effect upon spermatogenesis. From data secured by measuring the diameter of 25 consecutive tubules adjacent to the tunica albuginea, it would appear that the hormone caused a slight enlargement of the tubules of Fetuses 3 and 5.

The abundance of interstitial cells caused the tubules to be more widely separated than in controls, as may be seen in Fig. 1 to 4. Almost as convincing as the photographs (illustrating views observed by means of the micro-comparator) are data obtained as follows. A section of testis of an injected fetus was matched with that of its littermate control so perfectly that the 2 sections were virtually identical in shape, circumference and portion of testis from which they came. Each section was projected to a sheet of paper, it and its tubules were rapidly sketched and the sketches of tubules were counted.

Considering the several observations, it is assumed that little or none of the gonadotrophin traversed the placental barrier and that therefore it did not influence the testes of controls. It is likely that the hormone stimulated the testes of injected fetuses to a greater extent than indicated by the data since at autopsy it was noticed that these fetuses were somewhat smaller than controls. They, unlike controls, received such experimental insults as being shelled out of the amnion, transferred to the abdominal cavity of the mother and subjected to subcutaneous injections.

The response of the interstitial cells to injections of gonadotrophin suggests that normally the hormones of pregnancy influence the prenatal development of the testis. With respect to other experimental approaches to this problem, it is scarcely necessary to consider maternal hypophyseal gonadotrophin since it is probable that little or none of it crosses the placental barrier.[5] Also, one cannot conceive of a method of depriving the testis of hormones produced by the placenta; although essentially this condition prevails in marsupials, the testis of the opossum fails to react to injections of equine gonadotrophin until approximately the 63rd day in the pouch (judging from the prostate)[6] or the 17th day (judging from the bulbo-urethral glands).[7] However, the testis might be deprived of fetal hypophyseal gonadotrophin by hypophysectomizing the fetus, a project which is receiving the attention of the writer.

From microscopical studies of the ovary and volumetric determinations in which every fifth section was used, there was no evidence that the gonadotrophin caused this organ to grow or to undergo precocious cellular differentiation. Similarly, Price and Ortiz[2] noted no changes in the minute anatomy of the ovaries of rats which were injected after birth and killed on the sixth day of postnatal life.

Since the results of certain experiments in adult rodents suggest that the blood serum of pregnant mares contains not only gonadotrophin but also corticotrophic substances,[8,9] microscopical and volumetric studies of the fetal adrenals were made. In determining

2 Price, D., and Ortiz, E., *Endocrinology*, 1944, **34**, 215.

3 Hammar, J. A., *Z. f. angew. Anat. u. Konstitutionslehre*, 1914, **1**, 312.

4 Boyden, E. A., *Carnegie Inst. Wash., Contrib. to Embryol.*, 1940, **28**, 157.

5 Goodman, L., and Wislocki, G. B., *Am. J. Physiol.*, 1933, **106**, 323.

6 Moore, C. R., and Morgan, C. F., *Endocrinology*, 1943, **32**, 17.

7 Rubin, D., *J. Morph.*, 1944, **74**, 213.

8 Zalesky, M., Wells, L. J., Overholser, M. D., and Gomez, E. T., *Endocrinology*, 1941, **28**, 521.

9 Golla, Y. M. L., and Reiss, M., *J. Endocrinology*, 1942, **3**, 5.

volume, every tenth section was used. From these studies no evidence emerged that the hormone caused hypertrophy of the cortex or changes in the cortical cells.

In conclusion, injecting gonadotrophin under the skin of fetal rats resulted in an increase in the size and number of interstitial cells of Leydig. While it seems likely that these cells secreted more androgen than normally, positive evidence of this was not obtained. The injections failed to cause any significant changes in the ovary and the adrenal.

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Urinary Excretion of Thiamine as a Characteristic of the Individual.*

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The urinary excretion of thiamine has been used as a measure of the intake and of the body stores of this vitamin. That there is some relation between these functions is shown by the fact that an increase in the intake produces an increase in the excretion. It is usually assumed that individuals on the same intake will excrete approximately the same amount of thiamine. There are very few reports in the literature which provide detailed data for individuals who have been maintained for any length of time on a constant vitamin intake. Consequently the individual variation, and hence the reliability of the estimation of intake from individual excretion, is practically unknown.

In the course of investigations on the response of normal young men to various levels of thiamine intake,[1] a marked variation between the urinary thiamine excretion of different individuals was observed. The inter- and intra-individual variations in the urinary thiamine excretions are considerable at intake levels from 0.6 to 2.0 mg per day.[2] In spite of the great intra-individual variation, there was an indication that over a period of time, the thiamine excretion level was highly characteristic of the individual.

Experimental. Ten different menus were used consecutively in this work. They all contained the same amount of thiamine (0.50 ± 0.07 mg per day) and were reasonably constant and "normal" in composition of carbohydrate, fat and protein. We found that in man large increases in the dietary content of carbohydrates, fats or proteins have no appreciable influence on the dietary excretion of thiamine.[3] In order to determine whether the marked variations in the daily thiamine excretion were due to variations in the dietary content of foodstuffs other than the above, we fed our subjects the same diet on 3 successive days. Most of the items in these diets, including the meat, were canned or dehydrated. Where this was not the case, large quantities of the food items were secured at the start of the experiment to insure uniformity in the diet. All food was served in weighed portions. An exact duplicate of all foods served during the day was saved for thiamine analysis.

* Work done in part under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the University of Minnesota. Important assistance was also provided by the National Dairy Council operating in behalf of the American Dairy Association, Chicago, Ill., the Sugar Research Foundation, New York, N.Y., and the Nutrition Foundation, New York, N.Y., Merck and Co., Inc., Rahway, N.J., and Abbott Laboratories, North Chicago, Ill., provided a generous supply of vitamins. Most of the food materials were supplied by the Subsistence Branch, Office of the Quartermaster General, U. S. Army, and the J. R. Simplot Dehydrating Co., Caldwell, Idaho.

1 Keys, A., Henschel, A., Taylor, H. L., Mickelsen, O., and Brozek, J., *Am. J. Physiol.*, 1945, **144**, 5.

2 Mickelsen, O., Caster, W. O., and Keys, A., in press.

3 Mickelsen, O., Caster, W. O., and Keys, A., unpublished data.

TABLE I.

Thiamine excretion of normal young men maintained on the same diet for 3 days. The first 6 men received 1.0 mg thiamine per day while the others received 2.0 mg. All values for thiamine are expressed as μg of thiamine per day.

Subject	Urinary excretion				Stools
	Dec. 26	Dec. 27	Dec. 28	Mean	
A	118	75	79	91	540
Pa	36	21	39	32	510
E	60	48	53	54	800
M	85	83	86	85	550
S	55	46	49	50	720
R	62	52	60	58	666
Mean	69	54	61	62	631
D	276	294	359	310	1,090
C	82	87	88	86	490
B	240	141	158	180	1,240
W	128	193	109	143	960
Pe	114	128	98	113	1,840
H	148	122	138	136	—
Mean	165	161	158	161	1,124

The subjects in these experiments were 12 normal young men who had already been on the dietary regime for about 30 days. This length of time is required to secure a state of equilibrium in the urinary thiamine excretion.[2] Six of the men were given thiamine pills to raise their total daily intake to 1.0 mg while the other 6 were likewise increased to 2.0 mg. Placebos were given to make certain that all men received the same number of pills. The physical activity and mode of life for some time previous to and during this period were maintained reasonably constant. On each of the 3 days of this experiment, 24-hour urine samples were collected in bottles containing glacial acetic acid and toluol as preservatives. Stool samples, delineated by means of carmine, were also collected. The thiamine content of the urine and stools (total thiamine) was determined by our modification of the thiochrome technic.[4] Pyramin (the pyrimidine-like component of thiamine excreted in the urine) was determined by a modification of the yeast fermentation method for the estimation of thiamine.[5]

Results. The urinary and fecal excretions of thiamine are given in Table I. There is a considerable range in the urinary thiamine excretions of the men on each of the levels of intake. For the group on 1.0 mg of thiamine per day, the mean values range from 32 to 91 μg while for those on 2.0 mg they range from 86 to 310 μg. This variation in daily urinary thiamine excretion is not correlated with the fecal thiamine content. Najjar and Holt[6] suggested that the bacterial synthesis of thiamine in the lower gastro-intestinal tract could contribute to the body's requirement as shown by the absorption of thiamine from a retention enema. Using quantities of thiamine comparable to those normally excreted in the stools, Alexander and Landwehr[7] were unable to find any indications of thiamine absorption after a retention enema was given. Even if the thiamine synthesized by the bacteria in the lower gastro-intestinal tract is absorbed, there is no relation in our cases between the total thiamine content of the stools and the urinary excretion levels.

Within each group, there is a 2 to 3 fold variation in the thiamine excretion in spite of the constancy of thiamine intake. There is some overlapping of the urinary thiamine

[4] Mickelsen, O., Condiff, H., and Keys, A., *J. Biol. Chem.*, 1945, **160**, 361.

[5] Schultz, A. S., Atkin, L., and Frey, C. N., *J. Ind. and Eng. Chem., Anal. Ed.*, 1942, **14**, 35.

[6] Najjar, V. A., and Holt, L. E., Jr., *J. Am. Med. Assn.*, 1943, **123**, 683.

[7] Alexander, B., and Landwehr, G., *Science*, 1943, **101**, 229.

TABLE II.

Daily pyramin excretion of normal young men maintained on the same diet for 3 days. The first 6 men received 1.0 mg of thiamine per day while the others received 2.0 mg. All values for pyramin are expressed as μg of 2-methyl-4-amino-5-ethoxymethyl pyrimidine.

Subject	Urinary excretion				Stools
	July 23	July 24	July 25	Mean	
A	187	169	157	171	383
Pa	162	170	142	158	208
E	148	178	178	168	37
M	178	198	179	185	532
S	188	173	146	169	61
R	159	169	150	159	94
Mean	170	176	159	168	219
D	211	264	248	241	42
C	231	270	245	249	66
B	261	204	235	233	63
W	211	202	253	222	47
Pe	254	209	272	245	35
H	248	247	243	246	30
Mean	236	233	249	239	47

excretions in the 2 groups. The lowest mean value of the 2 mg group is equal to or less than the 2 highest figures for the 1 mg group. This indicates that it may be difficult in certain cases to distinguish from a single thiamine excretion value whether a person is receiving 1 or 2 mg of the vitamin.

In view of these findings, it was deemed advisable to repeat the above experiment. This was done after the men were maintained on their respective thiamine intakes for an additional 7 months. The conditions of the first experiment were duplicated again for a 3-day period.

The results of the thiamine excretion were essentially the same as on the first occasion of the experiment. This time pyramin was also determined. The pyramin content of the urines is given in Table II. There is considerably less variation in the urinary excretion of pyramin than is the case for thiamine. Furthermore, there is no overlapping in the excretion levels of the 2 groups. This is discussed at greater length elsewhere.[2] The fecal excretion of pyramin is on an average less than one-third that of thiamine when the 2 are reduced to molecular equivalents. Here again, there was no positive correlation between the fecal and urinary excretion of pyramin. Actually there was an inverse relation in the 2 groups, the group receiving 1 mg of thiamine per day showed a higher average pyramin excretion than the group receiving 2 mg.

The above experiment was followed by a period of 14 days on an essentially zero thiamine intake after which all 12 subjects were on routine diets for a period of one month. At this time the controlled thiamine regime was instituted again except that the levels of intake were reversed for the groups. Those who had been on 1.0 mg of thiamine now received 2.0 mg and vice versa. All other factors of diet and supplementation were the same as before. Twenty-four-hour urine samples were collected for thiamine analysis after the men had been on their respective new levels of intake for about 2½ months.

The gross ranking of the urinary thiamine excretions on the 3 above occasions was made with the results shown in Table III. The correlation coefficients for the gross ranking of the men has been calculated according to the method described by Snedecor.[8] Under these conditions the values above 0.81 are significant. In all of our cases, the rank correlations of the urinary thiamine excretions are significant. This indicates that the urinary excretion of this vitamin is intimately related

[8] Snedecor, G. W., *Statistical Methods*, 1946, Iowa State College Press, Ames, p. 164.

TABLE III.

Gross ranking of urinary thiamine excretion of normal young men on 2 levels of thiamine intake. The first 6 men were on 1.0 mg of thiamine from Nov., 1944, through July, 1945, and on 2.0 mg in Nov. and Dec., 1945. The other 6 were on 2.0 and 1.0 mg at these respective times. The thiamine excretions are in μg per day and are the averages of 3-day periods. The figures in parenthesis are the gross ranks of the men. The correlation coefficients are for the gross rankings of adjacent columns.

Subject	Dec., 1944		July, 1945		Dec., 1945
A	91 (1)		83 (1)		411 (1)
M	85 (2)		63 (2)		356 (2)
R	58 (3)		51 (3)		303 (3)
E	54 (4)		47 (4.5)		292 (4)
S	50 (5)		47 (4.5)		255 (5)
Pa	32 (6)		28 (6)		212 (6)
Mean	62		53		305
Rank correlation coefficient		0.99		0.99	
D	310 (1)		316 (1)		88 (2)
B	180 (2)		245 (2)		92 (1)
W	143 (3)		171 (3)		56 (3)
H	136 (4)		154 (4)		36 (5)
Pe	113 (5)		134 (5)		45 (4)
C	86 (6)		89 (6)		26 (6)
Mean	161		185		57
Rank correlation coefficient		1.00		0.89	

to some unknown characteristic of the individual.

This phenomenon did not occur on these 3 occasions only but was apparent throughout the entire course of the 8 months' experiment. The intra-class correlation was +.97 for the group receiving 1 mg of thiamine per day and +.85 for the group on 2 mg. Both of these values are significant.[2] The intra-class correlation is a measure of the consistency with which the various individuals in a group maintain their relative positions within the group throughout the entire experiment. This holds true whether the level of intake is increased or decreased. Those individuals who excrete large amounts of thiamine do so at various levels of intake. Additional evidence of this has been obtained from another experiment in which the thiamine intake was 5.0 mg. Three of the men in one of the above groups were subjects for this experiment. The gross ranking of the urinary thiamine excretion was the same as for the lower levels of intake.

There is no apparent explanation for the relationship between an individual and his level of thiamine excretion. A number of factors might be suggested. Fecal excretion as a measure of bacterial synthesis has been ruled out.[7] Under this category, we should also mention possible variations in the absorption of this vitamin. It is highly unlikely that there is any great variation in the absorption of thiamine in these cases. Physical activity does not account for the differences. In all experiments, a large share of the daily activity of these men was regulated by walks on a motor-driven treadmill. In the evenings and on Sundays, the physical activity was unrestricted. The most active men on off-hours are randomly distributed throughout the 2 groups. In separate experiments to determine the effect of severe physical exertion, there was no change in the thiamine excretion.[3] This has also been observed by Wang and Yudkin.[9] Differences in kidney function might be another possible explanation. No evidence is available on this point, but if this were the only factor then, over even a short period of time, the thiamine level in the blood of the "low excreters" should increase to such a point that they would gradually approach the "high excreters."

[9] Wang, Y. L., and Yudkin, J., *Biochem. J.*, 1940, **34**, 343.

There was a positive but non-significant correlation between the B.M.R. and the urinary thiamine excretion. The weights of these men ranged from 137 to 168 lbs. Here also, there is no correlation between weight and thiamine excretion. Variations probably exist in the way that these individuals metabolize thiamine. We know that only about 30% of the thiamine intake at these 2 levels can be accounted for as the sum of the urinary thiamine and pyramin excretions.[2] Breakdown products of thiamine other than pyramin probably vary inversely as the thiamine excretion.

No accurate prediction of the magnitude of one subject's excretion at a higher or lower level can be made from data of any one control period. For instance, in the 2 different groups D and A had approximately the same level of excretion on an intake of 1.0 mg per day whereas there was a marked difference in the excretion levels when the intake was 2.0 mg (313 versus 411). The overall excretion of the group on 2.0 mg thiamine in December 1945 was considerably higher than that of the other group when it was on this intake. The excretion of the subjects on 1.0 mg was approximately the same for both groups.

There are a few references to the variations of individuals on the same level of thiamine intake.[9,10] In these reports the great variation in the thiamine excretion is apparent but the data do not permit any more detailed analysis. In both of these reports, there is a strong suggestion that the thiamine excretions of their subjects are characteristic of the individuals. De Jong states that "The mean of the daily excretions of a weekly period is, for the same individual, more or less constant."[10, p 127]

Summary. 1. The thiamine and pyramin excretions by 2 groups of normal young men maintained on 1 and 2 mg of thiamine per day have been determined on 2 different occasions when the same diet was served on 3 successive days. Each of these periods occurred only after the men had been stabilized at their respective intakes.

2. The thiamine excretion on a constant diet shows a 2 to 3 fold variation for the men within each group and an overlapping of the 2 groups. The pyramin excretion is much more constant and shows no tendency for the values of the 2 groups to overlap.

3. The excretion of thiamine is characteristic of the individual as shown by the rank correlations of the excretion values. This was true over the entire 8 months of the dietary regime and was also true when the above thiamine intakes were reversed. There is no such correlation for the pyramin excretions.

4. The explanation of the thiamine excretion as a characteristic of the individual cannot be found in the fecal excretion of thiamine, the physical activity of the subjects, in the B.M.R. nor in their body weights.

[10] de Jong, S., *Arch. néerl. de physiol.*, 1940, **25**, 57.

15441

Persistence of Elevated Values for the Thymol Turbidity Test Following Infectious Hepatitis.*

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The thymol turbidity test introduced by Maclagan,[1] because of its simplicity and the ease with which results can be reproduced, is finding wide application in the study of patients with liver disease.[2,3] It is extremely

* The Bureau of Medicine and Surgery of the U. S. Navy does not necessarily endorse the ideas set forth in this paper.

[1] Maclagan, N. F., *Nature*, 1944, **154**, 670.

[2] Recant, L., Chargaff, E., and Hanger, F. M.,

TABLE I.
Values for Plasma Bilirubin, Bromsulphalein Retention, and the Thymol Turbidity Reaction in Patients 6 Months or More Following an Attack of Infectious Hepatitis.

	No. cases	% with increased plasma bilirubin	% with increased bromsulphalein retention	% with increased thymol turbidity values
Persistent symptoms	27	41	44	67
No symptoms	30	10	0	7

sensitive and may be of special use in evaluating mild symptoms that persist in patients who have had an attack of infectious hepatitis. A study of a group of cases presenting vague complaints more than 6 months after an attack of infectious hepatitis was made in the out-patient department of the Hospital of The Rockefeller Institute, and it was found that the thymol turbidity test was positive more often than any other test of liver function (Table I). It would seem to be of considerable importance, therefore, to determine the significance of this reaction. A study of the results of serial determinations of the thymol turbidity test throughout the course of infectious hepatitis, in cases with and without complications, has furnished certain information.†

In the acute stage of the disease values of the thymol turbidity test showed a delayed rise reaching a peak at least a week later than did values for plasma bilirubin and bromsulphalein retention, and at a time when symptoms of the disease had almost disappeared. These values then fell to normal more slowly than did those of any other test of liver function, several months sometimes being required for a return to normal. Fig. 1 illustrates the delayed rise and fall in the intensity of the thymol turbidity reaction in a typical case.

Approximately 18% of the cases of infectious hepatitis studied at the Hospital of The Rockefeller Institute developed definite recurrences during convalescence. These usually followed the first period of unrestricted activity and were characterized chiefly by an increase in bromsulphalein retention and a return of mild clinical symptoms. Values of the thymol turbidity test reflected all of these recurrences by a delayed rise one to 3 weeks after the onset of the recurrence. Extremely high values were sometimes found, frequently higher than those observed in the initial attack. These high values persisted for as long as 6 months after other objective signs of relapse had disappeared, and did not appear to give a true indication of the clinical state of the patient. Fig. 2 and 3 show the delayed rise and markedly prolonged elevation of the thymol turbidity values following typical recurrences during convalescence. In these cases, the second attack, although extremely mild, produced a more marked response in the thymol turbidity reaction than did the initial attack.

Results of the cephalin flocculation test paralleled the thymol turbidity values in most of the cases. Because quantitative measurements were more difficult, serial determinations of the cephalin flocculation test did not give curves that could be clearly interpreted. However, this test also remained positive for a greater period following relapse than after the first attack.

Certain patients with infectious hepatitis had marked persistence of symptoms and abnormalities in other tests of liver function for long periods without demonstrating definite recurrences; these cases showed only slightly

PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 245.

[3] Watson, C. J., Rappaport, E. M., *J. Lab. and Clin. Med.*, 1945, **30**, 983.

† Estimation of the thymol turbidity reaction was carried out by the method of Maclagan[4] modified for the use of the Coleman Jr. spectrophotometer; Evans blue dye (T-1824) was used as a standard of reference. A concentration of 3 γ per cc is equivalent to 20 thymol turbidity units. The Evans blue standard appears to have certain advantages over the barium sulfate standard previously employed in this laboratory.[5]

[4] Maclagan, N. F., *Brit. J. Exp. Path.*, 1944, **25**, 234.

[5] Shank, R. E., and Hoagland, C. L., 1946, **162**, 133.

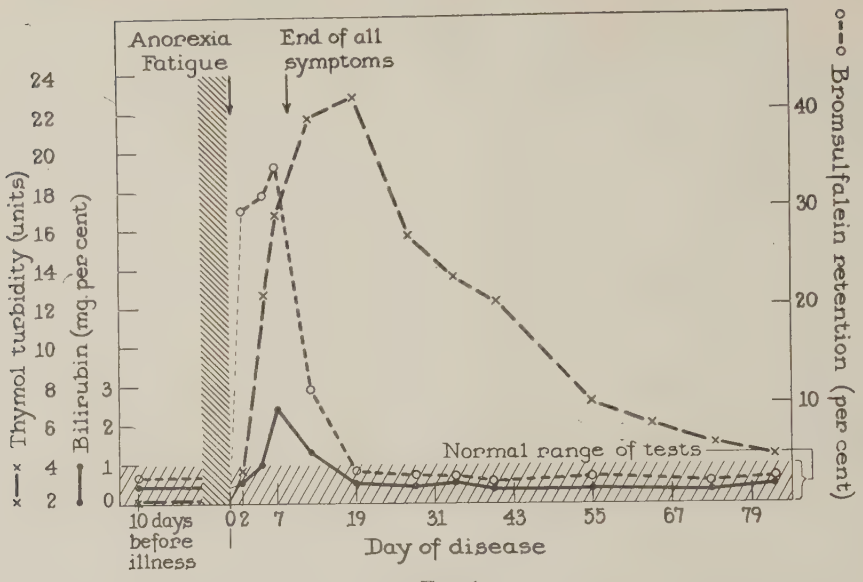

FIG. 1.

Comparative results on the serial determination of plasma bromsulfalein, the level of plasma bilirubin, and the thymol turbidity reaction of the serum in infectious hepatitis (mild uncomplicated case).

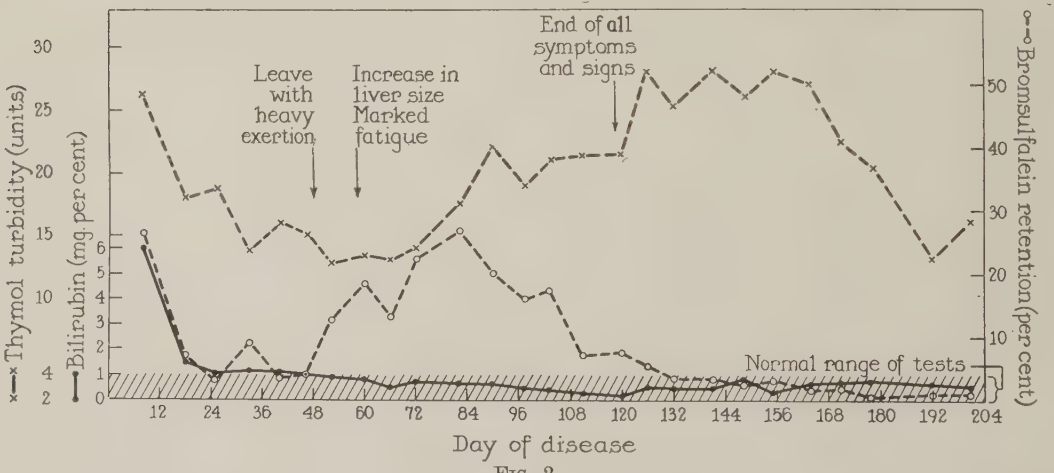

FIG. 2.

Comparative results on the serial determination of plasma bromsulfalein, the level of plasma bilirubin, and the thymol turbidity reaction of the serum in infectious hepatitis (recurrence during convalescence).

increased values for the thymol turbidity test. The greatest and most prolonged elevations were seen following recurrences of the disease and in these cases the values appeared to be out of proportion to the condition of the patient as judged by the mild clinical symptoms and the results of other tests of liver function. The significance of these high values is not clear. It may be that a selective function of the liver is measured which re-

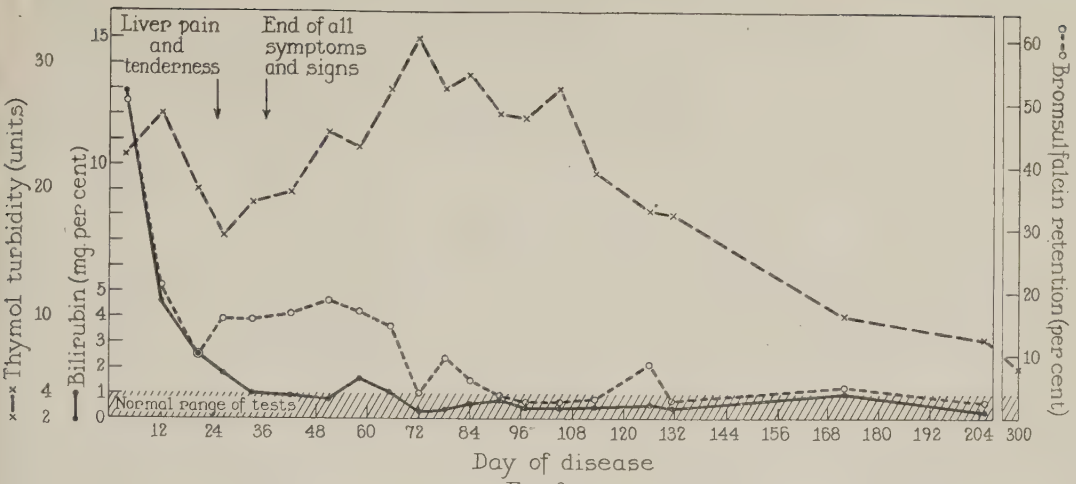

FIG. 3.

Comparative results on the serial determination of plasma bromsulfalein, the level of plasma bilirubin, and the thymol turbidity reaction of the serum in infectious hepatitis (recurrence during convalescence).

mains impaired for a prolonged period. The fact that these patients developed a recurrence of hepatitis shows that there was persisting activity. On the other hand, since the maximal response in the thymol turbidity reaction occurred coincident with the period of recovery following recrudescence, the possibility that the reaction is related in some manner to reparative phenomena in the liver should be entertained.

Studies of the protein precipitated by the thymol reagent are now in progress and the results may aid in understanding the significance of the reaction. At present it may be said that values of the thymol turbidity test should be interpreted with special care because they often appear to reflect previous events in a patient's illness. However, because of the simplicity and marked sensitivity of this test, it is very useful for following patients with liver disease, if the above limitations are kept in mind.

Summary. Values for the serum thymol turbidity test show a delayed rise and fall following an acute attack of infectious hepatitis. Recurrences of the disease produced a marked increase in the thymol reactivity of the serum which persisted long after other objective signs had disappeared. These findings indicate that the thymol turbidity reaction may show a delayed and prolonged response to liver injury.

15442 P

A Simplified Clinical Procedure for Measurement of Glomerular Filtration Rate and Renal Plasma Flow.*

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The measurement of discrete renal functions by means of "clearance" technics has been of great value in the study of both normal and abnormal kidney physiology. However, the technics[1] required for the accurate measurement of glomerular filtration rate, renal plasma flow and tubular function have several disadvantages, especially when applied to human subjects. Since inaccurate collection of urine constitutes the main source of error, an indwelling catheter, bladder washouts, and 3 or more serial collections of urine are required to yield reasonable accuracy. The amount of time devoted to the collection of blood and urine samples and to the chemical analyses prevents the standard method of measurement of the discrete renal functions from being a simple routine clinical procedure.

Several investigators[2-5] have devised technics which make the collection of urine unnecessary. These methods depend upon the rate of disappearance of inulin (or mannitol) and diodrast (or *p*-amino hippuric acid) from the blood after intravenous injection. These technics require serial blood samples to insure accuracy, and are not suitable for repeated observations at intervals of less than one day.

A simple procedure for the measurement of glomerular filtration rate and renal plasma flow has been devised that requires only a "blank" plasma sample and a single plasma sample for each estimation of the renal functions. This procedure is based on the assumption that, at equilibrium, the amount of a non-metabolized substance injected intravenously per minute should equal the amount excreted by the kidney per minute; *i.e.* providing this is the only excretory route. The measurement of glomerular filtration rate (inulin clearance) simply requires an intravenous infusion pump† that delivers fluid containing a known amount of inulin at a very constant rate, and a sample of plasma obtained after equilibrium between the rates of injection and excretion of inulin and its distribution in the body fluids is established. The clearance is calculated by dividing the amount of inulin injected per minute by the plasma inulin concentration. (Clearance equals

$$\frac{\text{Inulin excreted per minute, mg} \times 100}{\text{Plasma inulin concentration, mg per 100 ml}}$$

and at equilibrium, equals

$$\frac{\text{Inulin injected per minute, mg} \times 100}{\text{Plasma concentration, mg per 100 ml}})$$

The renal plasma flow (*p*-amino hippuric acid‡ clearance) can be measured simultaneously in the same way.

"Priming" intravenous injections of inulin and *p*-amino hippuric acid were given in amounts calculated, on the basis of their estimated volumes of distribution, to achieve plasma levels of 5 and 2 mg % respectively.

* This investigation has been aided by a grant from the Carnegie Corporation of New York.

1 Goldring, W., Chasis, H., Ranges, H. A., and Smith, H. W., *J. Clin. Invest.*, 1940, **19**, 739.

2 Barnett, H. L., Proc. Soc. Exp. Biol. and Med., 1940, **44**, 654.

3 Findley, T., and White, H. L., Proc. Soc. Exp. Biol. and Med., 1940, **45**, 623.

4 Newman, E. V., Bordley, J., III, and Winternitz, J., *Bull. Johns Hopkins Hosp.*, 1944, **75**, 253.

5 Landowne, M., and Alving, A. S., *J. Lab. Clin. Med.*, 1946, **31**, 453.

† The infusion pump used in these experiments consisted of a worm-driven rod that pushed on the plunger of a 50 ml syringe. This is excellent for short experiments, but not for any study extending over 3 or 4 hours.

‡ Since *p*-amino hippuric acid is acetylated by man it is necessary to determine "total" *p*-amino hippuric acid after hydrolysis.

TABLE I.

Glomerular Filtration Rate and Renal Plasma Flow as Measured by Infusion Pump Technic (A) and by Usual Clearance Technic (B).

Subject	Surface area (sq.m.)	Exp.	Time after start of infusion (min.)	Plasma inulin (mg %)	Glomerular filtration rate (ml/min) A	Glomerular filtration rate (ml/min) B	Plasma PAH (mg %)	Renal plasma flow (ml/min) A	Renal plasma flow (ml/min) B	Filtrate fraction % A	Filtrate fraction % B
An	1.80	1	70	4.44	130	129	1.62	659	618	19.8	20.9
			85	4.44	130	127	1.72	619	598	21.0	21.2
			99	4.47	129	122	1.78	598	570	22.6	19.9
					130	126		625	595	20.7	21.1
Br	1.93	2	67	5.29	145	161	1.46	899	942	16.1	17.1
			80	5.08	151	124	1.50	877	722	17.2	17.2
			90	5.19	148	155	1.56	835	806	17.7	19.2
					148	147		870	857	17.0	17.8
Ca	2.10	3	70	5.52	148	149	1.67	774	740	19.2	20.1
			83	5.52	148	152	1.68	770	798	19.2	19.1
			97	5.52	148	135	1.68	770	734	19.2	18.4
					148	145		771	757	19.2	19.2
		4	64	5.11	150	140	1.86	754	681	19.9	20.6
			80	4.87	158	152	1.79	784	742	20.2	20.5
			93	4.90	156	149	1.86	754	668	20.7	22.3
					155	147		764	697	20.3	21.1

The sustaining infusions delivered by the pump to maintain these levels were calculated on the basis of the estimated renal functions. Under these conditions, equilibrium was achieved with inulin shortly after 60 minutes and with *p*-amino hippuric acid within 30 minutes.

Glomerular filtration rate and renal plasma flow, as measured by the technics just described, were compared with simultaneous measurements by the usual method involving 3 serial urine collection periods. Four experiments in 3 normal young adult male subjects are summarized in Table I. Excellent agreement between the 2 technics was obtained when the averages of 3 periods are considered. The infusion technic, however, appears to yield more consistent individual values. The variations noted among the serial standard clearances are presumably the result of inaccuracies in urine collection.

Comment. A simple technic for the measurement of glomerular filtration rate and renal plasma flow has been devised. It requires a constant intravenous infusion pump and a minimum of blood samples and chemical analyses. It does not require urine collection and for this reason is simpler and more accurate. The method can be utilized for repeated serial observations during 24-hour periods.

Whether this technic can be adapted to the measurement of the maximal rate of tubular secretion or reabsorption of substances excreted only by the kidneys has not yet been established. It will also be necessary to determine how accurately the method can reflect acute changes in renal function and the effect of acute changes in the volume of body fluid compartments upon the measurements.

Finally, it may be noted that this method could be applied to the measurement of the "clearance" of other substances by other organs, provided the substance under study is excreted or metabolized only by the organ in question.

Acknowledgement is made to Doctor W. A. Feirer, of Sharp and Dohme Incorporated, for a generous supply of ampuls containing sodium para-aminohippurate.

15443
Reaction of the Rat Peritoneum to Acid Colloidal Pigments.

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It is now well known that many types of foreign particulate matter are rapidly removed from the peritoneal cavity; this is particularly true of substances in acid colloidal form. Part of such a material is discharged into the lymph stream while the remainder is taken up by macrophages from adjacent connective tissues. MacCallum[1] and others have studied the free cells which appear in peritoneal fluid under these conditions, but most investigators have devoted but little attention to the peritoneum itself. In these investigations, an attempt is made to follow changes in the peritoneum and to note differences in its regional response to various acid-colloidal pigments.

Materials and Methods. Four groups of inbred albino rats, 67 in all, were used in these experiments, each group being composed of individuals of nearly the same age (within one week); all animals weighed 150-190 g at the time of the first injection. One group was intraperitoneally injected with each of the following pigments: trypan blue, Biebrich's scarlet, India ink, and Chlorazol black E (Erie black GXOO). These materials were made up as 2% solutions in distilled water. Animals were given daily injections of 1-3 cc over periods of time ranging from 1-75 days; they were killed 18-24 hours after the final injection. Tissues were fixed in Bouin, Helly or Regaud's solutions, dehydrated in alcohol

[1] MacCallum, W. G., *Bull. Johns Hopkins Hosp.*, 1903, **14**, 105.

FIG. 1.

Peritoneum of the pelvic fat body. Note the thickening of peritoneum due to collection of free histiocytes and macrophages. The thickest portion (on the left) is completely denuded of mesothelial covering. Photomicrograph × 100.

All unretouched photomicrographs reproduced herein are from tissues of animals injected with Clorazol black E.

and dioxan, embedded in paraffin and sectioned at 6 micra. The sections were stained with hematoxylin-eosin or Mallory's triple combination.

Observations and Discussion. The microscopic features of normal peritoneum are already well known having been fully described by Seifert,[2] Webb,[3] and others. Histologically, it is composed of a continuous surface layer of mesothelium which is closely applied to a subjacent connective tissue lamina. This connective tissue layer may form an external (serous) covering for various abdominal organs or may be continued deeply into extra-peritoneal fat or areolar tissue. As will be indicated later, this extra-peritoneal connective tissue has the same reactive potentialities as the deeper layer of peritoneum proper.

It has been previously noted by Addison and Thorington[4] that the type of free cell response to various pigments is largely dependent on the material used. The present investigation indicates that the same is also true for histological reactions in the peritoneum itself. Of the pigments employed, least reaction followed administrations of Biebrich's scarlet; there were progressively greater responses in the order named, after injections of trypan blue, India ink, and Chlorazol black E.

When pigment solutions are introduced in large doses (2-3 cc per day), ascitic fluid almost invariably collects in the peritoneal cavity. This fluid is thick and syrupy in consistency and fresh smears indicate that it contains an abundance of pigment-filled

2 Seifert, E., v. Möllendorff's *Handbuch der mikroscopischen Anatomie des Menschen.*, 1927, **6**, 340.

3 Webb, R. I., *Am. J. Anat.*, 1931, **49**, 283.

4 Addison, W. H. F., and Thorington, J. M., *Anat. Rec.*, 1918, **14**, 467.

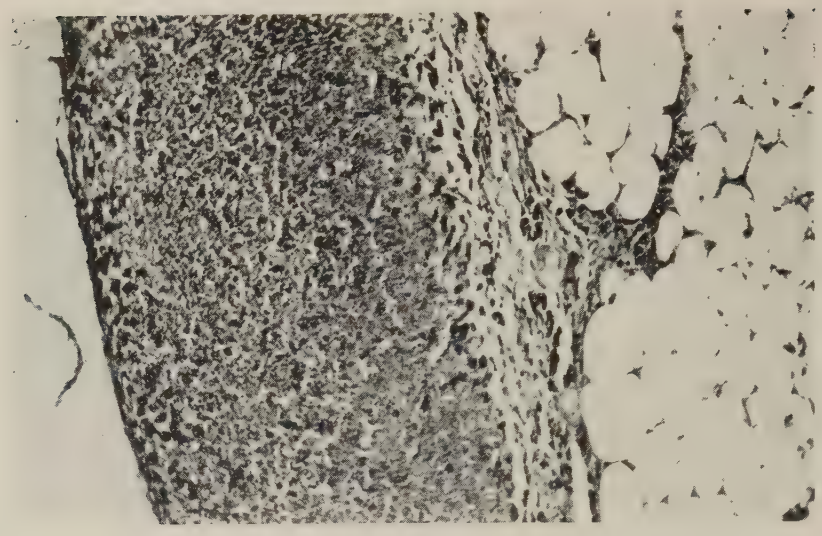

FIG. 2.
Mesentery, showing localized collection of free cells. In this mass there is a central necrotic area surrounded by a zone of intact macrophages. There is partial desquamation of mesothelium. Photomicrograph × 100.

macrophages, lymphocytes of all sizes, granulocytes, erythrocytes, and detached mesothelial cells.

Peritoneum as a whole reacts to injections of particulate matter by becoming colored and thickened; the degree of reaction is dependent on the type of material used and on the region examined. There is a minimum of response in that portion which forms serosa for organs of the gastro-intestinal tract; typically, its tissues are left almost undisturbed. The remaining peritoneum, however, is considerably thickened by collections of histiocytes and pigment-filled phagocytes in the sub-mesothelial connective tissue. Especially after chlorazol injections, the mesothelial cells round up, and, on occasion, separate from their fibrous bed. In such denuded regions peritoneal connective tissue is in direct contact with the peritoneal cavity and its contents (Fig. 1).

In almost all instances, pigment granules can be found within the mesothelial cell cytoplasm. The granules are of small size and rather uniform diameter. This is in sharp contrast to the inclusions of active phagocytes which appear in the sub-mesothelial connective tissue and in ascitic fluid. These macrophages contain pigment inclusions which are highly variable in size and number and which appear to be in all stages of coalescence.

If the experimental animals are subjected to daily injections for 2 or more weeks, some sub-mesothelial phagocytes will become distended by a single, densely colored pigment mass. Such condensation is never encountered in mesothelial cells, either in the attached condition or when they are found free in the peritoneal fluid. Particularly in the latter state, these free cells almost invariably show signs of disintegration. Cunningham[5] has noted that the ovarian germinal epithelium typically contains more granules than other portions of the mesothelial lining and believes that this pigment collection is phagocytic in nature. In these experiments, however, no signs of condensation or coalescence was seen in mesothelium from any region.

The mesentery reacts to injections of particulate matter in the manner described above, but presents several special features which might be mentioned in passing. More than in any other peritoneal region the mesenteric collections of sub-mesothelial phagocytes tend to become localized in nature. Sometimes these collections increase to considerable size

[5] Cunningham, R. S., *Am. J. Anat.*, 1922, **30**, 399.

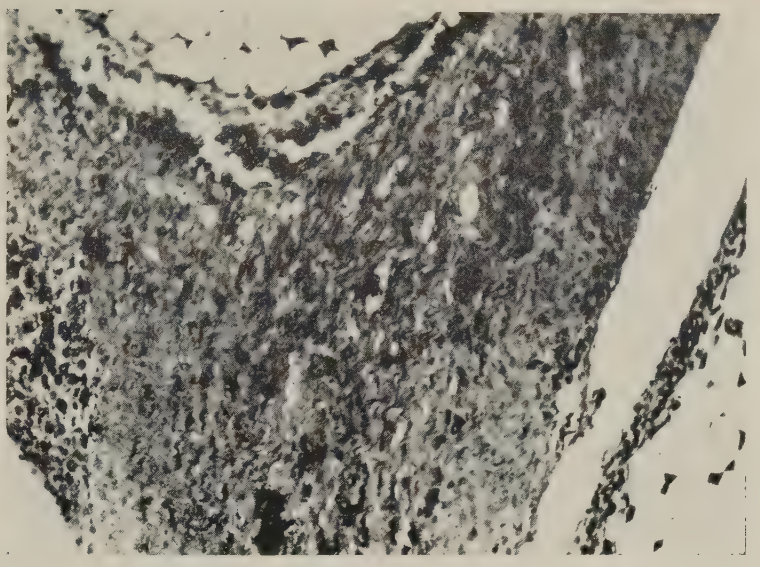

FIG. 3.
Omentum. Fibrous reaction in peritoneum. Note macrophages intermixed with the fiber bundles. Photomicrograph × 100.

and can be located grossly; in which case they are composed of a central necrotic mass of pigment granules and cell debris, surrounded by a zone of intact macrophages embedded in fibrous stroma (Fig. 2). This latter coat may become especially thickened by copious formation of granulation tissue.

The omentum of similarly treated rats has been previously described by Baillif,[6] but further investigations, particularly those employing injections of Chlorazol black E, have produced even more striking response than heretofore reported. Animals which are exposed to this dye for more than 2 weeks' time are prone to develop granulomatous masses; within these masses appear variable numbers of foreign-body giant cells which may or may not contain crystals. It is interesting to note that these crystals are not composed of the injected pigment but of an iridescent yellowish-green material of unknown composition. Occasionally, the chlorazol appears to excite a pronounced fibrous reaction which results in formation of firm, angular masses which are readily visible to the naked eye (Fig. 3).

The diaphragm shows particularly marked response to injections of colloidal pigments. As previously reported by Mackmull and Michels,[7] Simer,[8] and others, this musculofibrous membrane is an important site for removal of particulate matter from the peritoneal cavity. Especially after injections of India ink and chlorazol black E, the diaphragmatic lymph channels are markedly dilated, while the fibrous layer of peritoneum is thickened by collection of histiocytes and macrophages. No indications of stomata such as described by von Recklinghausen[9] and later by Allen[10] were seen in our preparations. Great numbers of mesothelial cells may become freed from the diaphragmatic peritoneum in response to acid-colloidal pigments. In this particular organ, islands of laminated mesothelium appear in the normally simple covering tissue; from these regions individual squamous elements are freed and discharged into the peritoneal cavity (Fig. 4).

The passage of pigment-filled macrophages

[6] Baillif, R. N., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 409.

[7] Mackmull, G., and Michels, N., *Am. J. Anat.*, 1932, **51**, 3.

[8] Simer, P. H., *Anat. Rec.*, 1944, **88**, 175.

[9] Recklinghausen, F. T. von, *Die Lymphgefässe und ihre Bebeihung zum Bindgewebe*, 1862, Berlin, Hirschwald.

[10] Allen, L., *Anat. Rec.*, 1936, **67**, 104.

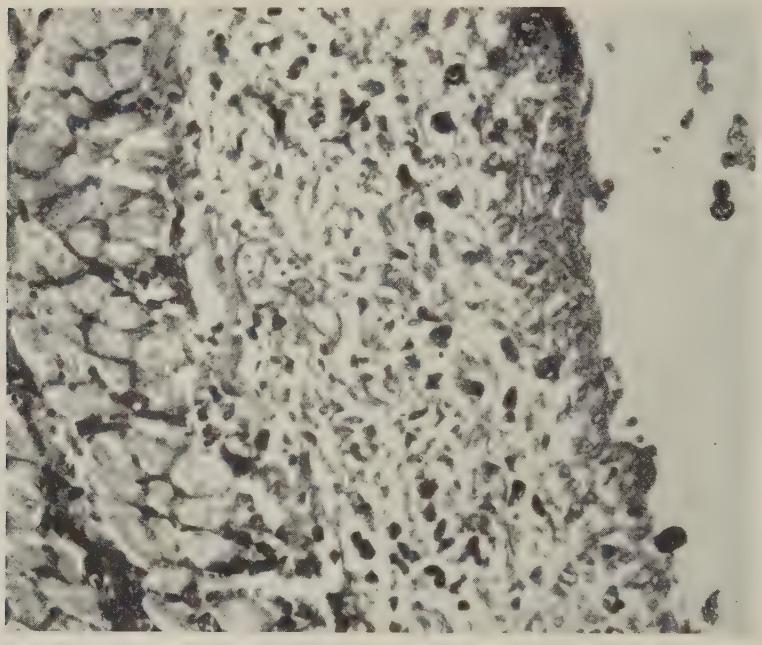

FIG. 4.

Section of the diaphragm. Mesothelium is lacking on a portion of the peritoneal surface (lower right); a macrophage can be seen traversing this denuded area. Other portions of its peritoneal surface appear to be covered by a laminated mesothelial covering (upper right). Macrophages and free mesothelial cells can be seen in the peritoneal cavity. Photomicrograph × 100.

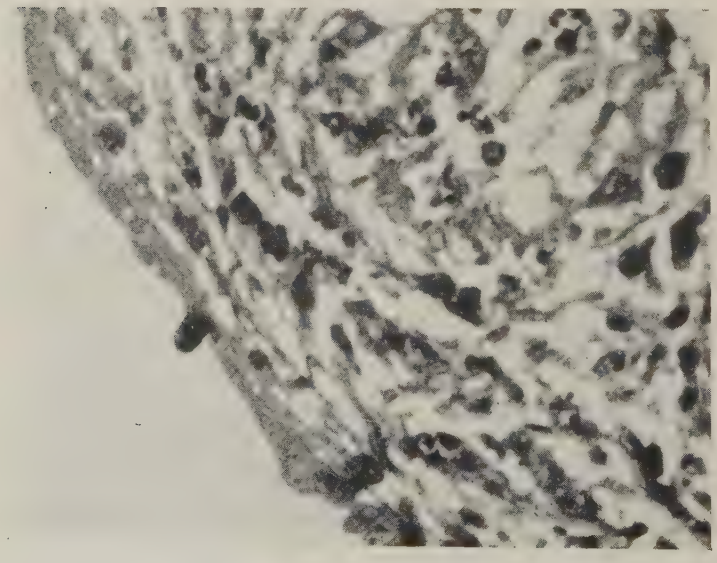

FIG. 5.

Mesentery. Note the collection of macrophages and histiocytes in the fibrous layer. In this case, the mesothelium is intact and a macrophage is passing between adjacent covering cells. Photomicrograph × 250.

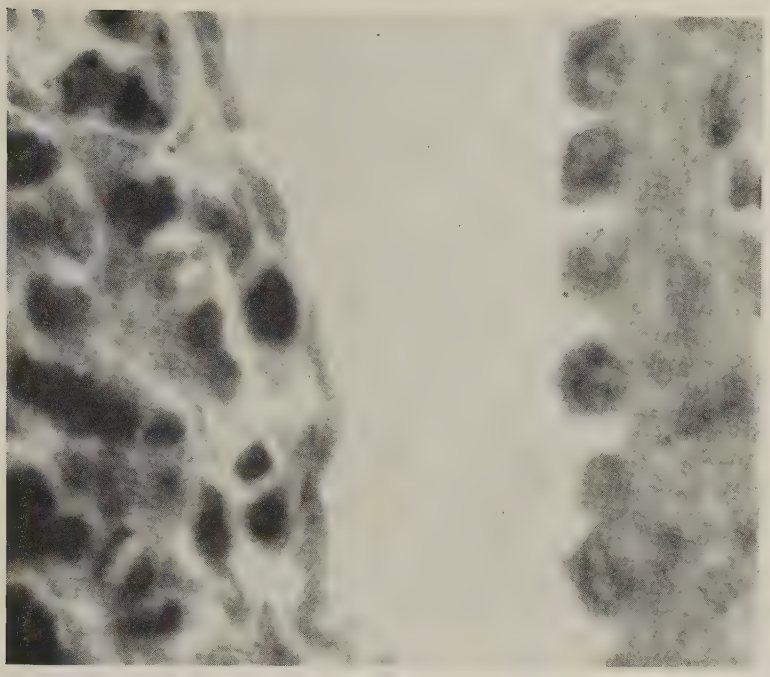

FIG. 6.
Portion of splenic capsule (on the right) and adjacent omentum (on the left of the photograph). Note the rounded mesothelial cells and lack of sub-mesothelial phagocytes in peritoneum of the splenic capsule, in contrast to squamous mesothelium and numerous macrophages in that of the omentum. Photomicrograph × 960.

through peritoneum is frequently seen. The majority escape from denuded areas such as those noted above, but the presence of an intact mesothelium does not offer insuperable barrier to their progress (Fig. 4 and 5).

The peritoneum covering organs of the gastro-intestinal tract is little affected by the presence of acid-colloidal pigments. Its surface layer remains a flattened mesothelium while the subjacent connective tissue lamina is but lightly infiltrated with macrophages. The peritoneum in relation to capsules of the liver and spleen is usually marked by formation of cuboidal mesothelial cells but there is little infiltration of phagocytes in the connective tissue lamina (Fig. 6).

Summary and Conclusions. Rat peritoneum reacts to the presence of various acid-colloidal pigments by thickening, primarily due to the accumulation of sub-mesothelial histiocytes and macrophages. The surface mesothelial cells may round up, and separate from their connective tissue bed. Pigment-filled macrophages escape through these denuded regions and pass into the peritoneal cavity; the same cells are sometimes seen to insinuate themselves between adjacent intact mesothelial cells as well. Localized collections of free cells may appear in the peritoneum of the mesentery and omentum; when large, these masses typically show necrotic centers.

15444

One Stage Functional Hepatectomy in the Rat.*

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The recent demonstration by Blakemore and Lord[1] of a simple and efficient method for the nonsuture anastomosis of blood vessels has allowed the development in the rat of a technic for indirect anastomosis of the portal vein with the inferior vena cava by way of the left renal vein. This establishment of a portal-caval shunt, coupled with ligation of the hepatic artery, results in the preparation of a functionally hepatectomized animal.

Method. Adult male rats of the Long-Evans strain are anaesthetized with ether. A long midline incision is carried from the xiphoid process to the symphysis pubis. The major portion of the gastro-enteric tract is exteriorized to the left of the supine animal to allow adequate visualization of the portal vein and inferior vena cava. The exteriorized viscera are kept warm and moistened with saline gauze packs. The left renal vein is brought into the operative field and stripped of adjacent fat. The suprarenal and gonadal veins are ligated at a distance from the renal vein. The renal artery is ligated close to the aorta, and is then stripped away from the renal vein. The renal vein is ligated at its point of exit from the kidney and the latter organ is then excised. A control ligature is placed about the renal vein at the point of entrance into the inferior vena cava to prevent reflux of blood into the renal vein. A small thin-walled pyrex glass sleeve of appropriate size is then threaded over the renal vein. Under the binocular dissecting microscope, the ligature is removed from the free end of the renal vein and the vein is then cuffed or everted over the glass sleeve and tied securely in place with a fine silk ligature. The portal vein and hepatic artery are then isolated from the neighboring structures in the hepatic pedicle and are ligated at their points of entrance into the liver. A control ligature is employed as a temporary block to prevent blood flow through the open portal vein at the time of anastomosis with the free end of the renal vein. This temporary ligation is most easily carried out by using a rubber band as a ligature, tension being brought to bear on the vein by fastening the stretched rubber with a small mosquito hemostat. A small incision is then made in the wall of the portal vein between the 2 ligatures, and into this aperture is introduced the glass sleeve with the everted free end of the renal vein. The portal vein is then tied securely around the glass sleeve and the intervening renal vein. In effect, then, the intima of the portal vein is in direct contact with the intima of the renal vein, the glass sleeve remaining entirely extravascular in position. The temporary ligatures about the portal vein and renal vein are removed allowing free circulation of portal blood into the vena cava via the left renal vein. The viscera are replaced in the abdominal cavity and the incision is repaired. The animals recover rapidly from the anaesthetic and appear normal to outward appearances for a number of hours, save for moderately diminished activity.

The glass sleeve employed has an external diameter of 1.5 mm, and a length of 2.5 mm, but these dimensions are varied in accordance with the variation in size of the vessels in any particular animal. A small constriction in the glass sleeve facilitates tieing the veins in place. The portal vein will generally accommodate a cannula with external diameter of 2 mm. It is unnecessary to heparinize the animal. The operation, if car-

* Assisted by grants from the Board of Research of the University of California and the Rockefeller Foundation of New York City.

1 Blakemore, A. H., and Lord, J. W., Jr., *J. A. M. A.*, 1945, **127**, 748.

ried out successfully, entails no blood loss. During the period of temporary portal vein obstruction, the intestines become markedly cyanotic, but normal color is regained immediately upon completion of the anastomosis and release of the temporary ligatures. The operation requires about 30 minutes. The major portion of this time is spent in preparing the renal vein and glass sleeve for insertion into the portal vein. The actual period of obstruction of the portal vein need not exceed 5 minutes.

If the hepatic artery be left patent, the rat will survive for an indefinite period with a complete portal-caval shunt (direct Eck fistula). These animals appear to be perfectly normal. Animals sacrificed 5 days after establishment of the portal-caval shunt show patent functioning anastomoses without evidence of portal obstruction. Secondary or delayed ligation of the hepatic artery may then be carried out at a later date.

Discussion. The survival time in a series of 12 unfasted adult rats averaged 11½ hours (range: 5-17 hours). The technic of nonsuture anastomosis of the portal vein to the inferior vena cava via the left renal vein obviates the use of a cannula directly in the bloodstream, and further does not require heparinization of the animal. The animals recover readily from the anaesthetic and seem normal for about two-thirds of the survival time, when they become progressively less active. Death usually occurred after a series of convulsive attacks. The animals were injected hourly after operation with glucose in saline solution by the subcutaneous route. At autopsy, the portal-renal anastomoses were patent. The liver was pale yellow in color and practically bloodless on incision. It is thought that the functionally hepatectomized rat or the rat with a portal-caval shunt is a useful preparation for experimental use.

Summary. A method is described whereby the portal vein is directly anastomosed to the left renal vein in the rat by a nonsuture method of blood vessel anastomosis. The complete direct Eck fistula produced in a one-stage operation coupled with ligation of the hepatic artery results in the preparation of a functionally hepatectomized animal. The survival time in a series of 12 rats thus prepared averaged 11½ hours.

15445 P

Viruses Inactivated by Mustard (*Bis*(β-chloroethyl) Sulfide) as Vaccines.

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For some years we have been using the rapidly advancing knowledge of protein chemistry in an attempt to find an agent other than formaldehyde that would inactivate viruses without destroying their antigenic nature. Formaldehyde reacts primarily with amino groups of protein but it also attacks other radicals. While some formalinized virus preparations are good immunizing agents many are not satisfactory, perhaps because the virus is not present in sufficient quantity. Reagents known to react with amino, tyrosine phenol, or SH groups of protein and several detergents have been used with disappointing results. Either the virus was not completely inactivated or else it failed to immunize animals to the active virus. It has been shown that mustard ($Cl—CH_2—CH_2)_2—S$ reacts with proteins[1] and it is our purpose here to report the tests we have made on the effect of this chemical on a number of viruses.

Eastern equine encephalomyelitis virus, fixed rabies virus, and hog cholera virus have been treated with chemically pure mustard.

[1] Berenblum, I., and Wormall, A., *Biochem. J.*, 1939, **33**, 75.

TABLE I.
Immunity Produced by Eastern Equine Encephalomyelitis Virus 2 Times Saturated with Mustard.

Immunizing injection 0.5 ml into right pad. Dilution used	Time after preparation and results			
	24 hr	2 wks	3 mo.	6 mo.
10^{-1}	0/4*	0/3	0/4	0/4
10^{-2}	0/2	0/3	0/4	0/4
10^{-3}	—	—	1/4	2/4
Controls	2/2	3/3	4/4	3/4

* Numerator—No. of animals that died.
Denominator—No. of animals inoculated.

For most of our experiments virus-containing suspensions were twice saturated, but in some instances they were half saturated by mixing an equal quantity of freshly saturated and centrifuged cold salt solution with the virus material. Care was taken to keep the reaction of the materials between pH 7 and 8 and at 25°C. Contrary to our previous results with other chemical reagents, we have had no difficulty in completely inactivating these viruses and all of the inactive preparations have had some immunizing ability.

Supernatants from suspensions of chick embryos, dead following inoculation with eastern equine encephalomyelitis virus, twice saturated with mustard are completely inactive and appear to be as good immunizing agents as any formalinized preparations. A typical experiment, given in Table I, shows that as little as 0.5×10^{-2} ml immunized guinea pigs regularly up to 6 months after its preparation. The material was injected into the right pad and the test for immunity was made by injecting suspensions of brain of infected guinea pigs into the left pad.

Fixed rabies virus in the form of suspensions of brain from infected guinea pigs, mice, and a dog has been treated and tested for its immunizing ability. Inactivation has been regularly obtained. In Table II are the results of one experiment in which brain from an infected dog was treated with different amounts of mustard and compared with vaccines prepared by adding 1% phenol and 1% chloroform. Guinea pigs were used for the experimental animals and received 2 intraperitoneal injections of 1% brain at an interval of 7 days. Two weeks after the last injection their immunity was tested by intramuscular injection of fixed rabies virus in guinea pig brain. The half saturated suspension gave complete immunity, while the once saturated material and the phenol or chloroform inactivated virus gave questionable protection.

TABLE II.
Immunity Produced by Fixed Rabies Virus.

Inactivation of vaccine	Results of intramuscular injection of active virus. Dilution	
	1/10	1/50
1 X saturated with mustard	4/4	1/4
1/2 saturated with mustard	0/12	0/11
Phenol treated	4/4	2/4
Chloroform treated	3/4	3/4
Controls	4/4	3/4

Hog cholera virus both in the form of sera from infected animals bled on the 4th day of fever and of organ suspensions has been used after two saturations with mustard. The test for immunity was made 3 weeks after the last injection by housing the animals with an infected pig. All of 9 control pigs died and showed typical lesions of hog cholera. Of 23 vaccinated pigs, 2 died from causes other than hog cholera, 3 died from acute hog cholera, and the remaining 18 showed some degree of immunity. All had an increased temperature; in one animal it lasted 1 day, in most of the others it lasted from 3 to 5 days, while in a few it persisted longer than this, but all survived the infection.

While there is much work yet to be done the results obtained show that mustard will inactivate the viruses tested and that the inactivated materials can be successfully used as vaccines.

15446

Experimental Chronic Hypertension in the Rat: Structural Changes in Blood Vessels, Heart and Kidneys.*

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Despite the frequent use of the rat in the study of experimental hypertension, microscopic studies of the organs of hypertensive rats are few.[1,2,3,4] Moreover, since in most of these earlier studies, animals manifesting acutely fatal symptoms,[2] dead of pyogenic infections,[3] or with suppuration[4] were examined, the observations are not applicable to uncomplicated chronic hypertension.

Experimental. Varying degrees of chronic hypertension were produced in 16 piebald rats (Evans-McCollum strain) by application of a figure-of-eight ligature to one or both kidneys.[5] The ligature was applied first to the right kidney of each rat when the animals were about 9 months old. Nine of the rats which failed to develop a rise in blood pressure to at least 150 mm of mercury within 3 or 4 months were then subjected to a similar operation on the left kidney. All of the animals were killed after the blood pressure had been maintained at levels between 150 and 240 mm of mercury for periods varying from 1 to 8 months. The blood pressures were determined by the plethysmographic method.[6] During this period of observation a gradual rise in blood pressure occurred. However, the final pressure as determined by daily readings had existed for at least 2 to 4 weeks, before the animals were sacrificed.

The heart, kidneys, spleen, liver, lungs, and occasionally other organs (pancreas, testis, brain, lymph node) were fixed in 10% formalin, imbedded in paraffin, and stained with hematoxylin and eosin. Microscopic examination of these organs and particularly of the heart and kidney disclosed the following pertinent data: Changes in the arteries of all the organs examined were minimal or absent. In every animal there was an increase in the width of the myocardial fibers of the left ventricle which in some instances were 2 or 3 times as wide as the fibers of the right ventricle. The striations of the fibers remained fairly intact. The nuclei were vesicular and retained their usual oval or "cigar" shape. Varying degrees of fibrosis occasionally accompanied these changes. The hypertrophy was usually commensurate with the degree and duration of the observed elevation in blood pressure.

In the kidneys, varying degrees of changes were observed involving individual or groups of nephrons. In the glomeruli, the changes ranged from slight focal fusion of the capillaries with Bowman's capsule to complete disappearance of the capsular space, increase of the connective tissue with hyalin change, and obvious obliteration of the structural pattern. The corresponding convoluted and collecting tubules contained a homogeneous bright pink material and were lined by flat cells. Their coarse convolutions occupied spaces larger than those of normal tubules. The widths of the cortical and medullary zones were decreased in proportion to the damage to the renal parenchyma. In focal areas there was an increase in the connective

* Aided by a grant from the John and Mary Markle Foundation.

1 Wilson, C., and Byrom, F. B., *Lancet*, 1939, **1**, 136.

2 Friedman, B., Jarman, J., and Klemperer, P., *Am. J. Med. Sci.*, 1941, **202**, 20.

3 Cromartie, W. J., *Am. J. Med. Sci.*, 1943, **206**, 66.

4 Smith, C. C., Zeek, P. M., and McGuire, J., *Am. J. Path.*, 1944, **20**, 721.

5 Grollman, A., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 102.

6 Williams, J. R., Harrison, T. R., and Grollman, A., *J. Clin. Invest.*, 1939, **18**, 373.

tissue stroma with some slight infiltration of lymphocytes, plasma cells and large mononuclear cells. The cotton thread with which the kidney had been constricted was demarcated by a zone of connective tissue with occasional giant cells of the foreign-body type about the constricting thread which was usually imbedded in the kidney substance. A fairly diffuse and almost uniform involvement was observed in all of the kidneys to which a ligature had been applied. Occasional single damaged nephrons were also observed in the kidneys not ligated. The structural changes in the two kidneys were always different. Without exception, the involvement was more marked and more extensive in the kidney to which a ligature had been applied than in the opposite kidney.

Discussion. The animals for these studies were selected from those manifesting a maximal degree of hypertension and were killed while still in good physical condition. In all instances, the hypertensive state was of sufficient duration to produce a marked cardiac hypertrophy particularly of the left ventricle. Renal lesions apparently responsible for the hypertension were observed in every kidney to which a ligature was applied. Focal lesions of a slighter degree apparently due to the hypertension were observed in the opposite intact kidney. Though there were marked alterations in both heart and kidneys, no pertinent changes of any degree were observed in the large or small arteries of the heart, kidneys, spleen, liver, pancreas, lungs and brain. The inflammatory changes described by Cromartie[3] and these noted by Smith, Zeek, and McGuire[4] are explained by the fact that most of the animals used by Cromartie[3] died of pyogenic infection and suppurative lesions were common in those used by Smith *et al.*[4] The necrotic vascular lesions noted by Wilson and Byrom[1] were associated with an acutely developing severe hypertension and a rapidly fatal course. The findings of these authors are thus not applicable to chronic hypertension.

Summary. Varying degrees of chronic hypertension were produced in 16 rats by application of a figure-of-eight ligature to one or both kidneys. The animals were killed after the blood pressure had been maintained between levels of 150 to 240 mm of mercury for periods varying from 1 to 8 months. Microscopic examinations disclosed in each animal a marked cardiac hypertrophy particularly of the left ventricle. Renal lesions apparently responsible for the hypertension were observed in every kidney to which a ligature was applied and focal lesions of a slight degree apparently due to the hypertension were observed in the opposite intact kidney. No pertinent changes of any degree were seen in the large or small arteries of the organs examined.

15447

Effect of Quick Freezing at Very Low Temperatures of Donor Tissue in Corneal Transplants.*

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There are 2 concepts regarding the necessity of using living instead of dead tissue in corneal transplantation. Castroviejo[1] and many others believe that living corneas must be used if successful, clear transplants are to be obtained. Others, Löhlein[2] and Salzer[3]

* The work described in this paper was done under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Columbia University.

1 Castroviejo, R., *Am. J. Ophth.*, 1941, **24**, 1.

2 Lohlein, W., *Klin. Mon. f. Augenkeilk.*, 1938, **101**, 106.

3 Salzer, R., *Archiv. f. Ophth.*, 1921, **105**, 469.

4 Weiss, P., and Taylor, A. C., PROC. SOC. EXP.

have the opinion that corneal grafts are invaded and replaced by the corneal cells of the host, the graft serves only as a framework or bridge, which temporarily guides and supports the corneal cells of the host and need not be living tissue. This group then regards the corneal graft as analogous to the nerve bridge graft in the manner in which it serves. An experiment using frozen corneas as donor tissue would be a test, though not conclusive, of the concept that corneal grafts need not be living.

Materials for nerve bridge grafts have been prepared by freezing, followed by dehydration, Weiss and Taylor.[4] These authors also transplanted frozen-dried rat corneas,[5] with promising results. Their grafts, observed for 6 weeks post-operatively, were clear but "resettled by host cells in supra-normal numbers."

Rapid freezing of donor eyes in liquid nitrogen also appeared to be a hopeful method of preservation of corneas for transplantation because it was thought that tissue frozen quickly at very low temperatures suffers a minimum of physical and chemical change.[6,7] If grafts of frozen tissue were unsuccessful it did not seem likely that drying, storage, and rehydration, as done with nerve grafts, would add to their chances of success.

Corneal transplants were made in rabbits using the technic described by Castroviejo.[1] The grafts were of the full thickness of the cornea and 5 mm square. The control series consisted of 22 cases in which the donor corneas were from normal animals sacrificed one to 2 hours prior to the transplantation and were therefore grafts of living tissue. In the experimental series, corneas were used which had been frozen in iso-pentane chilled with liquid nitrogen. Iso-pentane was used because of the observations of Gersh[6] that bubbles of gaseous nitrogen formed about tissue immersed in liquid nitrogen, thus slowing down the freezing process. Eight eyes were frozen in chilled iso-pentane, and transferred to liquid nitrogen within 15 to 30 minutes; 6 others were frozen directly in liquid nitrogen. The shift from iso-pentane was made because some of this compound clung to the tissue until it was transplanted, and it was thought that it might be an irritant and delay healing. However, no difference was detected in the behavior of grafts from donor eyes frozen in the 2 media, so the 2 groups may be considered as one.

The donor eyes were obtained as in the control series and frozen by plunging them into the freezing medium. These eyes remained in the liquid nitrogen for one or 2 hours, except in 4 cases in which they were stored 3 to 4 days. In every case the eyes were removed from the liquid nitrogen to room temperature and the graft cut within a few minutes, as soon as the cornea thawed sufficiently. At the time of cutting, the aqueous humor and the rest of the eye remained frozen, which was necessary in order that the globe might retain its shape, and the cornea be firm enough to cut. Cutting the transplant at this stage of thawing was much easier than in the living eye. All of the cuts could be made with a knife which left desirably smooth, straight edges. The grafts were absolutely clear and normal in thickness; however, they rapidly became thick and edematous when their cut edges came in contact with physiological saline solution. The grafts fitted the prepared opening in the host corneas perfectly. The frozen corneas were as good or better than fresh corneal material in so far as operative procedure was concerned. The corneal epithelium, however, loosened readily on these grafts and undoubtedly was soon lost.

The eyes of both the control and experimental animals were closed with a simple lid suture which was cut on the fourth day. In the control group, one animal died within 6 days following the operation. In 9 others the transplant either became dislodged or failed to heal satisfactorily. Failure to heal well appeared to be due to poor fit of the graft which was noted at the time of the operation. Gross infections did not occur. Twelve transplants were clear (Fig. 1) and have remained so to this writing, 6 to 7½ months later.

BIOL. AND MED., 1943, **52**, 326.

5 Weiss, P., and Taylor, A. C., *Anat. Rec.*, 1944, **88**, 49 (Suppl.).

6 Gersh, I., *Anat. Rec.*, 1932, **53**, 309.

7 Hoerr, N. L., *Anat. Rec.*, 1936, **65**, 293.

Fig. 1.
Transparent corneal graft 4 months after operation. The donor tissue was fresh, living cornea.

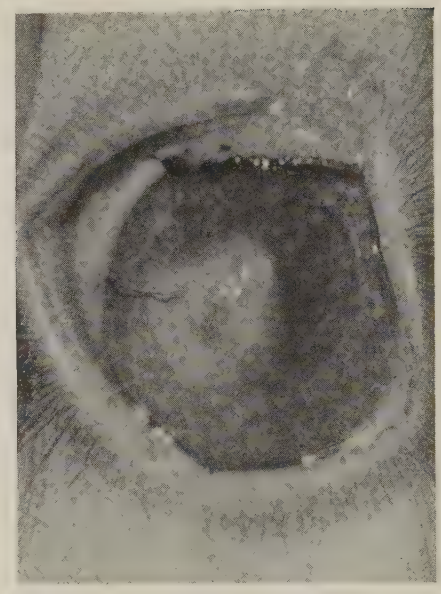

Fig. 2.
Corneal transplant 4 months after operation in which the donor tissue had been frozen in isopentane chilled with liquid nitrogen.

In the experimental group of frozen corneas the grafts were uniformly clear, healing well and as good generally as living grafts on the fourth day and therefore appeared hopeful. On the fifth, sixth, or seventh days the grafts become hazy, edematous, and the interface between graft and host became more distinct, deepening into a slight groove. The host tissue did not always become hazy, but blood vessels invaded the host cornea from the scleral border, a distance of about one millimeter. In several cases the center of the graft remained clear, while the peripheral portions became more and more hazy and edematous. The second week post-operative was a very difficult period in the healing of the graft, which appeared to behave almost as a foreign body. The grooves around the transplant were sometimes deep and it may be that in some a fistula formed, thus opening the anterior chamber for a second time. If this occurred it would account for the several synechias which formed in this group, though but once in the control series. All grafts of frozen tissue were opaque by the end of the second week and were becoming vascularized by the vessels which crossed from the sclera through the clear host cornea. The extent of this vascularization subsided somewhat by the fourth or fifth week, but all transplants were opaque and retained a slight blood supply. Six weeks to 2 months after the operation the eyes were not inflamed, the grafts were perfectly healed, the normal curvature of the cornea was restored, but the grafts were translucent or opaque (Fig. 2).

Discussion. The use of frozen corneas prepared in the manner described, for transplantation does not appear advisable. Their failure to persist as a transparent tissue suggests that efforts to preserve corneal tissue should be directed toward keeping the tissues alive for the necessary time. Freezing corneas in the manner described here appears to preserve the tissue in as nearly normal a condition as possible. The corneas were clear and not changed structurally but presumably they were no longer living. Certainly their structure as a bridge or framework would not be impaired by freezing. This procedure did not in any way make the technic of transplantation more difficult. At the time of operation and for 4 days thereafter these grafts appeared to be as good as grafts of living tissue.

It appears, therefore, that for the production of enduring, clear, corneal transplants,

viable tissues should be used. This implies that the stroma of clear, corneal transplants retains its identity as donor tissue, and is not replaced by the host within a short period. It may be that after some length of time some of the corneal cells die and are replaced by new cells derived either from the host or sister cells of the transplant. This process would only be that which may occur in a normal cornea to maintain the ordinary structure of the corneal stroma. Mitosis is exceedingly rare in the corneal stroma, but has been observed in colchicine-treated amphibian eyes (Peters[8]). This replacement may occur slowly, in rabbit eyes, as is suggested by the behavior of a few of the grafts in which the degree of opacity became reduced after 70 days, somewhat as the opacity of a heavy bond paper is modified by a drop of oil. The cornea did not become clear throughout its thickness and the improvement does not appear to be continuing at this time.

Summary. 1. Corneas frozen in liquid nitrogen can be grafted in rabbits, but invariably become opaque. Transplants of living corneal tissue, using the same technic as in the frozen, are successful and remain clear. 2. The use of frozen tissue is no handicap in the performance of the operation. 3. The results support the hypothesis that for the production of permanently clear corneal transplants living tissue must be used. 4. The data also support the suggestion that the corneal stroma of a clear graft retains its identity as donor tissue for a long period of time.

8 Peters, F. J., *The Cytological Effect of Colchicine on the Cornea of* Triturus viridescens *and of Sulfanilamide on* Allium cepa, thesis, 1946, Fordham University.

15448 P

Fibrinolytic Activity in Blood Serum During Pregnancy Complicated by Hypertensive Toxemias.

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The recent report by Smith and Smith[1] concerning fibrinolytic activity in the serum from pre-eclamptic and eclamptic patients and its absence in those with hypertensive disease with a superimposed pregnancy, suggests that there is a specific substance in the blood of the former groups which may be produced as a result of the toxemia. Should this prove to be true it would give a definite indication as to the direction toward which future research concerning the etiology of these conditions should be pointed and might well be utilized as a diagnostic test in certain obscure cases in which differentiation between the 2 conditions could not easily be made. In view of the fact, however, that the same activity was demonstrated in serum taken on the first day of menstruation and in post-operative patients, it seems more likely that this is a non-specific activity related to factors other than the pre-eclampsia.

In an effort to confirm this observation and to evaluate its application as a diagnostic procedure, bloods from a group of pregnant patients were tested for fibrinolysis.

Material. The patients were all attending the prenatal clinics of the Chicago Lying-in Hospital, the normal group being seen in the general clinic and the abnormal groups in the toxemia clinic. The normal patients demonstrated no signs which could be interpreted as indicating the presence of pre-eclampsia, with the exception of 2 who showed an excessive gain in weight. Those classified as

1 Smith, O. W., and Smith, Geo. Van S., *Science*, 1945, **102**, 253.

TABLE I.
Fibrinolysis in Serum from Normal and Complicated Pregnancies.

		Fibrinolysis—24 hr					
	No. cases	0 (?)	+	++	+++	++++	+++++
Normal	32	28	2		2		
Pre-eclampsia	10		1		3	5	1
Hypertension	30	14	3	9	4		
Abruptio placenta	2			2			
Thrombocytopenic purpura—pregnant	1				1		
Chronic glomerulonephritis—not pregnant	1		1				
Nephrosis—not pregnant	1				1		
1st day menstruation	18	2			8	6	2

having hypertensive disease had all had elevated blood pressures throughout the present pregnancy and in some instances had had previous hypertensive pregnancies in this clinic. Those diagnosed as pre-eclampsia had an onset of the classical signs of this condition appearing late in pregnancy without demonstrable residual upon post-partum examination. The bloods taken on the first menstrual day were from normal young women employed in various capacities in the hospital.

Method. To 0.8 cc of the fresh test serum in a 13 mm tube, 0.2 cc of normal plasma of proven clotting ability was added. After a stable clot had formed, the tube was incubated at 37°C for 2 hours, at which time the amount of dissolution of the clot was noted and 12 Upjohn units of thrombin were added; another reading of dissolution was made at the end of 24 hours. In the early cases various dilutions of test serum were used, but when it became evident that this procedure only demonstrated less fibrinolytic activity as the amount was diminished, only the one dilution was set up. The amount of clot destruction was graded from + to +++++, in which the latter indicated complete disappearance of clot and the former only a slight increase in fluid content in the tube.

Results (Table I). *Normal patients.* Of the 32 normal patients, all formed a firm initial clot which at the end of 2 hours showed no dissolution in 26 instances. In the remaining 6 there was questionable dissolution in 5 instances and ++ in one. One of these clotted completely with the addition of thrombin and showed no further dissolution in 24 hours, while of the other 5 none formed additional clot when thrombin was added; 2 showed +++, 2 +, and one questionable clot disintegration in 24 hours. Both patients with +++ dissolution had had an excessive gain in weight at the visit on which blood was drawn.

Pre-eclampsia patients. The bloods from all 10 patients with pre-eclampsia had definite fibrinolytic activity; one +, 3 +++, 5 ++++, and one complete dissolution. The patient showing a + reaction had a very mild toxemia, while the remainder had definite well developed pre-eclampsia which in one instance was classified as severe. No eclamptics were studied.

Hypertension. Of 30 patients with chronic vascular renal disease complicating pregnancy, 12 showed no clot dissolution and 2 a questionable change. In the remaining 16, 3 had +, 9 had ++, and 4 had +++ clot destruction. Dissolution was much less pronounced than in the pre-eclamptic series; however, 15 of those in which clot destruction was noted were similar to the pre-eclamptics in that they showed proteinuria and in 8 instances edema. The total amount of protein excreted in the 24 hours during which the blood was drawn varied from 0.2 to 1.8 g and the edema from plus 1 to plus 2. The degree of lysis was apparently not related to the amount of protein excreted, however, since the urine of the patients with +++ dissolution contained the smallest amount of protein. One patient without proteinuria or edema showed a ++ reaction, 2 showed

questionable reactions, and the remainder no clot destruction.

Menstruation. Of 18 bloods drawn on the first day of seemingly normal menstruation, 2 showed no lysis, 8 +++, 6 ++++, and 2 dissolved completely.

Miscellaneous. Two patients with abruptio placenta showed ++ lysis, one non-pregnant patient with chronic glomerulonephritis showed +, one child with nephrosis +++, and one pregnant patient with thrombocytopenic purpura +++.

Discussion. The results of the study of this group of patients indicate that fibrinolytic activity is absent in the normally pregnant patient but may be present when certain complications arise. That this ability to destroy clot is not associated with any specific complication is suggested by the fact that it was present in association with a variety of conditions. The fact that fibrinolysis could be demonstrated only in those patients in whom chronic vascular renal disease was associated with proteinuria and edema suggests that this condition closely resembles pre-eclampsia, in which fibrinolysis is uniformly present. In view of these findings it becomes evident that this test cannot be utilized as a means of accurately differentiating between vascular renal disease and the specific pregnancy toxemias.

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Effect of Minerals on Susceptibility of Swiss Mice to Theiler's Virus.*

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The more recent developments in our knowledge of nutrition have made it possible with some species of animals to use diets composed only of highly purified components. As a result it has been possible to study the role of certain specific nutritional factors in susceptibility to infectious diseases. Previous reports from this laboratory[1-3] have concerned the effect of vitamin deficiencies on the resistance of mice and monkeys to experimental poliomyelitis. The general biological importance of calcium, phosphorus, sodium, potassium and other minerals led us to undertake the following experiments on the effect of single deficiencies of these minerals on the susceptibility of Swiss mice to Theiler's GDVII virus.

Methods. The complete synthetic diet was composed of the following parts per 100: sucrose 73, vitamin-free casein 18, salts IV 4, and corn oil 5. The vitamin supplements per 100 g of diet were: thiamine 0.3 mg, riboflavin 0.3 mg, pyridoxine 0.3 mg, niacin 0.5 mg, calcium pantothenate 2.0 mg, *i*-inositol 100 mg, choline 300 mg, *p*-amino benzoic acid 100 mg and biotin 5 μg. The several mineral-deficient diets were produced by supplying the corresponding salt mixture from Table I instead of salts IV at the percentage indicated in that table. The phosphorus-low diet was produced by replacing the casein with blood fibrin and by giving the phosphorus-free salt mixture. All of the mice received distilled water and an adequate amount of oleum percomorphum *per os* each week.

Twenty-one- to 24-day-old Swiss† mice

* These studies were aided by a grant from The National Foundation for Infantile Paralysis, Inc.

1 Rasmussen, A. F., Jr., Waisman, H. A., Elvehjem, C. A., and Clark, P. F., *J. Infect. Dis.*, 1944, **74**, 41.

2 Lichstein, H. C., McCall, K. B., Elvehjem, C. A., and Clark, P. F., *J. Bact.*, in press.

3 Lichstein, H. C., Waisman, H. A., McCall, K. B., Elvehjem, C. A., and Clark, P. F., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 279.

TABLE I.
Composition of Salt Mixtures for Mineral Deficient Rations.* †

	Salts IV	Sodium	Potassium	Calcium	Magnesium	Phosphorus	Chloride
$CaCO_3$	1200	630	475	—	1200	1325	1200
$CaHPO_4$	248	248	1484	—	248	—	248
K_2HPO_4	1290	1290	—	1290	1290	—	1290
$NaH_2PO_4 \cdot H_2O$	—	—	—	200	—	—	—
K_2CO_3	—	—	—	—	—	1020	—
$MgSO_4 \cdot 7H_2O$	408	408	408	408	—	408	408
$NaHSO_4$	—	—	—	—	198	—	—
NaCl	670	—	670	670	670	670	—
Na_2CO_3	—	—	—	—	—	—	615
$CaCl_2 \cdot 2H_2O$	—	830	—	—	—	—	—
$FeC_6H_5O_7 \cdot 5H_2O$	110	110	110	110	110	110	110
KI	3.2	3.2	—	3.2	3.2	3.2	3.2
NaI	—	—	2.9	—	—	—	—
$MnSO_4 \cdot 4H_2O$	20	20	20	20	20	20	20
$CuSO_4 \cdot 5H_2O$	1.2	1.2	1.2	1.2	1.2	1.2	1.2
$ZnCl_2$	1.0	1.0	1.0	1.0	1.0	1.0	—
$ZnSO_4$	—	—	—	—	—	—	1.2
Total (gm)	3951.4	3541.4	3172.1	2703.4	3741.4	3558.4	3896.6
% of salts in ration	4.0	3.6	3.3	2.8	4.0	3.6	4.0
Control ration (addition per kilo)		6.1 Na_2CO_3	10.2 K_2CO_3	13.5 $CaCO_3$	1.4 $MgCO_3$	15.0‡ $NH_4H_2PO_4$	6.0 NH_4Cl

* All mice received distilled water.
† These salt mixtures were calculated by James H. Shaw.
‡ 15 g of $NH_4H_2PO_4$ supplies 50% more P than the amount in optimum diet. This is necessary to compensate for the amount of phosphorus present in 18% casein but which is not present in fibrin.

bred in our laboratory and split litter technic with consideration for sex and weight were used in all experiments.

The GDVII strain of Theiler's virus[3] was given by intracerebral injection of 0.03 ml in a 1% concentration. Uninoculated mice were maintained on both deficient and optimal diets for control purposes and to determine the most desirable time for virus administration, so that the end of the incubation period in the mouse should occur at the peak of the deficiency. After inoculation the mice were observed twice daily.

Results. Potassium. On this diet with potassium-free salts, young mice failed to grow and developed signs of unkempt fur, general weakness, apathy, extreme inactivity and anorexia. The mice lost 1-1.5 g during the first week and generally maintained this weight up to 21 days, although in some instances additional weight losses up to 1.0 g were recorded. Following this period the mice lost weight rapidly and died during the fourth week.

† Original source was the laboratory of Dr. Webster.

When 5% of the potassium level in salts IV was supplied, the mice lost only 0-1.5 g during the first week but slowly gained up to their initial weights, during the following 2 weeks. These animals developed the same signs as the mice fed diets devoid of potassium, but the mortality rate was considerably lower. When 15% of the potassium level in salts IV was supplied the mice gained up to 5.0 g during the first 14 days, and then either gained weight slowly or maintained their body weight. These mice appeared normal and no deaths occurred in this group. When 30% or 100% of the potassium level in salts IV was supplied the mice gained 3.5-5.0 g per week during the 4-week experimental period and were normal in all respects. A total of 447 mice was employed in potassium studies.

In Table II are presented the data on the percentage of paralyzed animals in the various groups of potassium-deficient mice. Those fed diets devoid of potassium showed a marked resistance to the invasion of this virus (32% paralyzed), but a progressively decreased resistance was seen as the potassium level was increased to the optimal level (91% para-

TABLE II.

Influence of Level of Potassium and Sodium in Diet on Resistance of Swiss Mice to Theiler's GDVII Virus.

Series No.	No. of days on ration	No. of days after inoc.	% of mice paralyzed				
			K def. (34)*	5% of opt. K (35)	15% of opt. K (33)	30% of opt. K	K opt. (34)
57	8	5	0	14	15		26
		6	18	26	36		56
		7	21	37	58		74
		8	27	46	73		85
		9	29	51	79		91
		10	32	51	79		91
			(22)			(30)	(27)
54	13	5	0			3	4
		6	0			13	15
		7	0			13	15
		8	0			27	48
		9	5			47	56
		10	5			60	70
		11	9			67	78
		12	14			70	78
			(19)				(19)
62	10	6	16				53
		7	32				68
		8	32				84
		9	32				89
		10	37				95

Series No.	No. of days on ration	No. of days after inoc.	% of mice paralyzed		
			Na def. (18)	Na opt. (18)	Na-K def.
57	36	7	15	15	
		8	31	54	
		9	31	62	
		10	31	69	
		12	46	85	
		14	46	92	
		15	54	92	
			(24)	(26)	(27)
68	30	6	4	27	11
		7	30	54	11
		8	39	62	11
		9	43	65	11
		10	69	85	18
		13	69	88	18

* Numerals in parenthesis indicate number of mice inoculated.

TABLE III.
Influence of Level of Phosphorus in Diet on Resistance of Swiss Mice to Theiler's GDVII Virus.

Series No.	No. of days on ration	No. of days after inoculation	% of mice paralyzed			
			5% of optimum P (18)*	15% of opt. P (25)	30% of opt. P (24)	opt. (25)
61	15	5	17	8	38	4
		6	17	24	42	12
		7	28	32	46	36
		8	28	36	50	64
		9	33	44	54	72
		10	33	48	58	80
		13	33	48	58	80
			(21)	(19)	(21)	(21)
67	11	5	29	21	19	19
		6	38	26	24	19
		7	43	32	38	43
		8	48	32	48	67
		9	48	37	57	76
		10	48	42	67	86
		11	48	42	67	90
		12	48	42	71	90
		15	48	42	76	90

* Numerals in parenthesis indicate number of mice inoculated.

lyzed). The results clearly indicate that the incidence of paralysis varied directly with the potassium level of the diet.

Sodium. When mice were fed diets containing sodium-free salts they grew poorly and developed rough coats in 2-3 weeks, but were normal in other respects. The average weight gain for the first week was 2.0 g; during the following 3 weeks the animals did not gain and then began to lose weight slowly. There were no deaths in mice receiving this diet until the 6th week at which time the mortality was high. Control animals received diets containing an amount of sodium equivalent to that in salts IV. These animals gained 4.0 g per week and were normal in all respects.

A concomitant deficiency of sodium and potassium was produced by feeding for 21 days a diet containing a salt mixture which was free of sodium and contained only 15% of the potassium supplied by salts IV. After 21 days, all of the potassium was removed from the diet and by 30 days the mice had developed signs of the multiple deficiency of sodium and potassium. These signs were similar to those seen in mice fed potassium-low diets. The addition of potassium at a level of 15% of the optimal was found to be necessary to prevent the high mortality of a potassium deficiency, while producing a sodium deficiency which commonly required about 4 weeks.

Mice fed diets devoid of sodium showed a somewhat lowered incidence of paralysis than control groups which had received adequate amounts of sodium (Table II). However, when mice were fed diets lacking both sodium and potassium they showed a marked resistance to the invasion of this virus. This resistance was comparable to that shown by mice fed diets lacking only potassium (Table II).

Phosphorus. A phosphorus deficiency was produced in mice by feeding a synthetic diet containing a phosphorus-free salt mixture (Table I) and blood fibrin in place of casein. The animals on this diet grew poorly, averaging a gain in weight of 2-4.5 g during the 4-week period with the major portion of this gain in the first 7-10 days. The deficient mice had a flabby appearance, developed unkempt fur, a rapid and shallow type of respiration and a flaccid paralysis of the hind legs which upon gross examination was indistinguishable from poliomyelitis. When this

TABLE IV.
Influence of Calcium, Magnesium and Chlorine in Diet on Resistance of Swiss Mice to Theiler's GDVII Virus.

Days after inoculation	% of mice paralyzed					
	Ca deficient (26)*	Ca optimum (13)	Mg def. (11)	Mg opt. (20)	Cl def. (19)	Cl opt. (14)
4	0	0	27	25	0	0
5	12	7	45	50	0	0
6	25	23	45	70	0	0
7	44	23	63	75	0	0
8	69	69	63	75	0	7
9	75	77	63	75	16	14
10	75	84	63	75	32	29
11	75	84	63	75	68	50
12	75	84	63	75	68	64
13	75	84	63	75	89	71

* Numerals in parenthesis indicate number of mice inoculated.
Ca—Mice on diet 36 days before inoc.
Cl " " " 49 " " "
Mg " " " 20 " " "

diet was modified to contain that amount of phosphorus (as $NH_4H_2PO_4$) supplied by salts IV and casein in the complete diet, it supported excellent growth (3.5-5.1 g per week) and the mice seemed normal in all respects.

When the amount of phosphorus in the diet was less than that supplied by the complete diet, the animals grew in proportion to the amount of phosphorus supplied. The unkempt fur, rapid respiration and flaccid paralysis of the hind legs no longer developed when 5-15% or more of the phosphorus was supplied in the complete diet. As it was impossible to distinguish between paralysis due to phosphorus deficiency and that due to virus invasion, when phosphorus was completely absent from the diet, we found it necessary to add an amount of $NH_4H_2PO_4$ which was equivalent to 5% of the phosphorus level in the complete diet. Under these conditions the mice did not develop an extreme phosphorus deficiency and flaccid paralysis of the hind legs due to phosphorus deficiency was seldom seen, unless the experimental period was extended to 6 weeks.

As the amount of phosphorus was increased, the per cent of paralyzed animals in the groups of mice receiving these diets likewise increased up to 80-90% in mice fed complete diets (Table III).

Calcium. Mice fed calcium-low diets grew slightly below the normal rate for the first week and then gained even more slowly during the following 4-5 weeks, but the only manifest signs of deficiency were unkempt fur and loose stools. Apparently a severe calcium deficiency did not follow when this diet was used.

However, in a small preliminary experiment (Table IV) no difference occurred in the percentage of paralyzed mice between groups maintained on calcium-low and on complete diets.

Magnesium. When a magnesium-free salt mixture was incorporated into an otherwise complete diet and fed to mice, the animals appeared normal and gained 3 g per week, whereas those fed the same diet with added magnesium gained 4-5 g per week over the same 4-week period. A few mice on the deficient diet did show unkempt fur and occasional evidence of peripheral vasodilatation as manifested by red ears.

There was no difference in susceptibility to Theiler's virus between mice fed diets low or adequate in magnesium.

Chlorine. Our complete diet with a chloride-free salt mixture replacing salts IV did not precipitate a chlorine deficiency in mice, even though care was taken to supply the choline requirements (0.3%) as choline in place of choline chloride.

However, the mice on chloride-low and on complete diets were inoculated with Theiler's virus after a 49-day depletion period. Both groups of mice showed the same incidence of

paralysis after administration of the virus (Table IV).

Summary. The influence of the level of 6 common minerals on the susceptibility of Swiss mice to Theiler's GDVII virus has been investigated in more than 1100 animals.

No demonstrable effect on the susceptibility to this infection was found by varying the level of calcium, magnesium or chlorine in the diet, some effect was noted with sodium, while striking results were obtained with potassium and phosphorus.

A progressively decreased resistance was observed as the amount of potassium or of phosphorus was increased in the diets up to the optimal levels.

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Influence of Various Pharmacologic Substances on the Emetic Effect of Intravenous Glutamic Acid in Dogs.

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The intravenous administration of amino acid mixtures of protein digests has become a promising method to aid in maintaining nitrogen balance in patients unable to take adequate food by mouth. A side effect, however, which imposes certain practical limitations is the occasional incidence of nausea and vomiting, particularly when the solutions are administered too rapidly. The chief offender was found to be glutamic acid (Madden *et al.*,[1] and Unna and Howe[2]). Aspartic acid has been shown to have a similar effect (Unna and Howe,[2] Madden *et al.*[3]) in dogs. Other reports have described undesirable side reactions when amino acids or protein hydrolysates were infused rapidly in humans. Although glutamic acid was not specifically mentioned, it is highly probable that in many cases it was largely responsible for the emesis.

With the knowledge that glutamic acid is a predominant offender, an attempt has been made to find drugs which will delay or inhibit emesis resulting from the infusion of a pure solution of the natural (*l*+) glutamic acid. Achieving this might materially enhance the clinical application of protein hydrolysates.

Methods. Healthy mongrel dogs maintained on a standard chow diet were used. Food was not withheld until 2 to 3 hours before the experiment. A 5% solution of *l*+ glutamic acid (not neutralized) having a pH of 1.1 was infused intravenously at a rate of 10 mg/kg/min until vomiting occurred. The substance to be tested as an inhibitor of emesis was either injected shortly before and/or during the glutamic acid infusion, or mixed with the solution. In order to establish individual tolerance, each dog was subjected to an infusion with glutamic acid alone, several days before or after the combined use of glutamic acid and the compound investigated. Any animal failing to vomit with glutamic acid alone was eliminated from the series.

Experimental Observations. Data recorded in Table I for *first-run* dogs show that animals given glutamic acid alone vomited when an average of 136 mg/kg had been administered at the relatively rapid rate of 10 mg/kg/min. Animals tested again within a 4- to 6-day period were found to have developed an increased tolerance (Table II). Therefore, in statistical comparison, it was necessary to use results on "first-runs" only. A rest period of 2 weeks to a month was

[1] Madden, S. C., Woods, R. R., Shull, F. W., Remington, J. H., and Whipple, G. H., *J. Exp. Med.*, 1945, **81**, 439.

[2] Unna, K., and Howe, E. E., *Fed. Proc.*, 1945, **4**, 138.

[3] Madden, S. C., Bassett, S. H., Remington, J. H., Martin, F. J. C., Wood, R. R., and Shull, F. W., *Surg., Gynec., and Obst.*, 1946, **82**, 131.

TABLE I.
Effect of Drugs on Vomiting Threshold of Intravenous Glutamic Acid.

		Drug	Dose	No. of dogs	Mean emetic dose mg/kg	Std. error	"P" value rel. to I
I	5% glutamic acid—10 mg/kg/min	None		20	136	8.26	
II	Same	Pento-barbital Na	2 mg/kg	17	241	20.8	<0.01
III	Same, with —	Epine-phrine	10 μg/cc infus. sol'n	10	198	18.3	<0.01

TABLE II.
Vomiting Threshold in Successive Tests, Showing Rising Tolerances.

Dog	First test mg/kg	Subsequent test mg/kg	Time between tests days
1	153	180	6
3	173	286	6
4	113	132	6
5	170	135	6
6	123	127	6
7	87	113	4
14	126	164	6
16	58	124	6
20	145	166	6
21	116	128	6

found to eliminate any acquired tolerance, so that tests on the same dog after such an interval were regarded as first-run results also.

Pretreatment. (a) *Atropine.* Three dogs (6 to 9 kg) were tested with this drug. The first received 3 mg of atropine sulfate intravenously 12 minutes before infusion of glutamic acid, and 2 mg after 8 minutes of infusion. The second was given 3 mg atropine intravenously 13 minutes before infusion, and the third received 3 mg atropine intraperitoneally 8 minutes before infusion. None of them showed a significant increase in tolerance to the emetic effect of glutamic acid.

(b) *D-desoxyephedrine.* Eight dogs each received *d*-desoxyephedrine, 1 mg/kg intravenously, 10 minutes before glutamic-acid infusion. No significant increase in tolerance occurred.

(c) *3,5,5-Trimethyloxazolidine - 2,3 - dione, (Tridione).* This drug was tested on 2 dogs. One received 25 mg/kg intravenously 2 minutes before the infusion of the glutamic acid. The second was given 50 mg/kg 2 minutes before the infusion, and another 50 mg/kg was injected into the tubing after 14 minutes of infusion. Neither showed a significant increase in tolerance.

(d) *Pyridoxine with thiamine.* One dog was given 50 mg pyridoxine and 100 mg thiamine intravenously immediately before the glutamic-acid infusion. The second received a similar dose and in addition 3 doses of 25 mg pyridoxine plus 50 mg thiamine were injected during the infusion period. The third was treated similarly but only 2 additional injections were made during the infusion. The fourth dog received 50 mg pyridoxine and 100 mg thiamine intravenously 15 minutes before the infusion. None of the 4 dogs reacted with a significant increase in tolerance.

(e) *Pentobarbital sodium (Nembutal).* Twenty "first-run" experiments comprised this series with a dose of 2 mg/kg nembutal administered intravenously 10 minutes before glutamic acid infusion started. Table I indicates the results. All animals, without exception, tolerated an increased amount of glutamic acid before vomiting occurred. The average amount to produce vomiting was 241 mg/kg, an increase of 84% over the control level. The nembutal injection produced no detectable sedation in the dog. Four experiments were carried out with 4 mg/kg nembutal. This amount also had no sedative effect, but the increase in tolerance was not appreciably greater than with the smaller dose. In one dog, where the infusion of glutamic acid was delayed 30 minutes after the injection of

nembutal, the protective action had virtually disappeared.

(f) *Epinephrine.* During the course of the experiments both with and without nembutal premedication, it was noted that certain animals could not be induced to vomit or required far greater than average amounts of glutamic acid before vomiting occurred. In most instances this was observed in dogs which were nervous, whining or struggling. It occurred to us that the augmented release of epinephrine in these animals might be a factor in the increase in tolerance. It was decided to carry out a series of tests in which the glutamic-acid solution would contain an amount of epinephrine which normally does not produce a significant rise in blood pressure in the unanesthetized dog. The quantity used was 2 μg of epinephrine per kg per minute. It was mixed in the infusion fluid to provide 10 μg epinephrine per cc of 5% glutamic acid, which resulted in a simultaneous infusion of each substance at the desired rate. Table I shows that the addition of such an amount of epinephrine to the infusion fluid produced an increase in tolerance to glutamic acid of nearly 46% (10 dogs), the average amount of glutamic acid required to produce vomiting being 198 mg/kg. Throughout the experimental series, occasional testing of blood and urine failed to show any evidence of hemolysis.

To determine the possible effects on blood pressure and pulse rate, direct recordings from the femoral artery were made on 4 unanesthetized dogs with a Hamilton membrane manometer. Pulse rates varied less than 10% under any of the experimental conditions. Blood pressure likewise was only slightly affected. This was true of the infusion of glutamic acid either with or without epinephrine. Variations of less than 20 mm Hg on either side of the control readings were obtained, and in the same dog successive tests showed opposite responses. Respiration was not affected. In none of the experiments was the infusion continued to the point of vomiting, in order that the complicating factors incident to this reflex would be avoided. No significant hyperpnea was noticed.

Results on dogs anesthetized with phenobarbital were markedly different with respect to circulatory effects. Glutamic acid alone infused at the usual rate of 10 mg/kg/min produced a marked fall in blood pressure, which was maintained as long as the infusion continued, and the recovery was gradual. Epinephrine with the glutamic acid produced a rise of 40 to 80 mm Hg which diminished slightly as infusion progressed, but never returned to normal until the infusion was terminated. Moderate hyperpnea resulted with epinephrine, while none occurred with glutamic acid alone.

Discussion. The average dose required to produce vomiting (136 mg/kg) with glutamic acid alone, differs from values obtained by others, who used a slower rate of infusion with a neutralized solution,[2] or a mixture of other amino acids along with the glutamic acid.[1] In the present experiments it might be expected that a faster rate would not allow as much time for escape of the glutamic acid from the circulation by any route, and thus the blood concentration would be expected to rise more rapidly. It might be pointed out that the development of a temporarily increased tolerance was strictly avoided and only "first-run" data were used in the statistical analysis of results. The number of animals used in the comparison of first and second runs was not sufficient to warrant statistical analysis, but only one of 10 dogs showed a decreased tolerance on the second run, while an appreciable rise in the threshold to vomiting occurred in all the others (Table II).

The choice of the various substances tested was based in part on an anticipation of their possible use in clinical practice, where little time is available for premedication before the infusion of the nutritive materials is started. The use of pyridoxine and thiamine for several days previously might give entirely different results, but such an experiment would not represent the usual clinical possibilities. Failure of atropine to produce an improvement indicates that the emetic response to glutamic acid is not exclusively parasympathetic. Tridione, as an anticonvulsant with little sedative action, was used to investigate possible cortical involvement. *D*-desoxyephedrine

as a central nervous system stimulant provided another avenue of approach.

The mechanism by which nembutal increases the tolerance to glutamic acid cannot be established with certainty from the present experiments. The drug may exert a depressant action directly upon the vomiting center or elsewhere along the nervous pathways, thus delaying the onset of the reflex by elevating the threshold. Another possible action would be a depression of the motility of the gastro-intestinal tract, which has been observed both *in vivo* and *in vitro* by Gruber and Gruber.[4,5] Shaw[6] has shown the site of depressant action to be both in the postganglionic fibers and in the muscle cells themselves.

The effect of epinephrine may be due to sympathetic predominance causing suppression of the vomiting reflex, with the action either central or peripheral, or both. The simultaneous administration of epinephrine, as outlined above, possesses the theoretical advantage that its action is limited to the actual duration of the infusion, because of its rapid inactivation in the body, whereas other agents given beforehand may either exert their effect still for some time after the need for them is ended, or may have such a brief effective period that a careful timing is necessary to take full advantage of the protective action. However, the physiological considerations involved in the administration of epinephrine might impose restrictive limitations upon its clinical usefulness.

From the results of 111 experiments obtained with these various agents, it would appear that the emetic effect of glutamic acid may be prevented or ameliorated in several possible ways. The administration of a suitable sedative, prior to the infusion of protein hydrolysates or amino acid mixtures prone to produce vomiting, might be attempted clinically in order to improve tolerance. This could be done either by injection or by rectal suppository.

Summary. 1. *l*+ glumatic acid, when infused at a rate of 10 mg/kg per minute in dogs induced vomiting on an average when 136 mg/kg had been administered.

2. Atropine, *d*-desoxyephedrine, tridione, or pyridoxine with thiamine had no significant effect in delaying vomiting.

3. When nembutal, 2 mg/kg was given intravenously 10 minutes before the glutamic-acid infusion, vomiting was not induced until an average of 241 mg/kg had been given, an 84% improvement in tolerance.

4. Epinephrine, infused with glutamic acid in a concentration of 2 μg/kg/min delayed vomiting until an average of 198 mg/kg had been infused, a 45% tolerance increase.

5. Tolerance to glutamic acid increased if infusions were repeated within 4 to 6 days.

6. The possible applications of these findings to the clinical use of protein hydrolysates are briefly discussed.

4 Gruber, C. M., Jr., and Gruber, C. M., *Arch. Int. Pharmacodyn.*, 1939, **63**, Fasc. 2, 243.

5 Gruber, C. M., and Gruber, C. M., Jr., *J. Pharm. and Exp. Therap.*, 1941, **72**, 176.

6 Shaw, F. H., *Austral. J. Exp. Biol. and Med.*, 1942, **20**, 117.

15451 P

Experimental Acceleration of Secretion of Urine in Fetal Rats.*

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In the fetal rat, ligation of the ureter causes the development of hydroureter and hydronephrosis, while ligation of the urogenital papilla causes the storing of urine† in the

* Aided by a grant from the medical research funds of the Graduate School.

† In collaboration with Dr. Gerald T. Evans and Miss Harriet Lamberg, the chemical composition

TABLE I.
Effects of Urea and of Ligating Renal Pedicles of Mother Upon Rate of Secretion.

Fetuses (male) Group	No.	Treatment at 2 hr preceding autopsy: Papilla	Injection of fluid under fetal skin	Body wt at autopsy g	Urine in bladder mg	mg/g
			Renal pedicles of mother not ligated			
A	8	Not ligated	0	4.1570	6.1	1.5
B	11	Ligated	0	4.1968	19.0	4.5
C	3	"	0.15 cc normal saline	4.5461	20.5	4.5
D	4	"	0.15 " distilled water	4.0568	20.7	5.1
E	6	"	0.15 " aq. sol. urea (500 mg/cc)	5.1504*	46.2	9.0
F	2	"	0.15 " " " " (150 " ")	4.7261	25.7	5.4
G	2	"	0.04 " " " " (150 " ")	4.5779	17.8	3.9
			Renal pedicles of mother ligated for period of 24 hr			
H	11	Not ligated	0	3.0679	53.4	17.4
I	3	Ligated	0	3.2652	56.1	17.2
J†	4	"	0	3.0873	33.1	10.7

* Marked edema at site of injection must have contributed to the body weight.
† Before ligating papilla, urine already in bladder was removed by means of a micro-pipette.

bladder.[1] The rate of secretion of urine, especially during the last 2 days of gestation, is much more rapid than studies in certain mammals suggest.[2]

The present account deals with attempts to speed up the rate of secretion by introducing urea into the fetal circulation and by causing metabolites to accumulate in the maternal circulation (ligation of renal pedicles of mother). Using a method recently described[3] and ether for anesthetizing the mother, each fetus except those in Groups A and H (Table I) was transferred to the abdominal cavity of the mother. The outlet of the bladder was blocked by ligating the urogenital papilla. The fetuses in Group J were subjected to laparotomy before ligating the papilla; by means of micro-pipette prepared for the purpose, the bladder was punctured and the urine already present was removed. After completing the several steps, the abdominal incision of the mother was closed (by suturing) and ether was discontinued. Subsequently, exactly 2 hours after tying off the papilla, each fetus was secured by severing the umbilical cord and killed by decapitation (decapitated at about the 16th hour before expected parturition or, in other words, at the end of the 21st day after observation of coitus). The bladder was dissected, removed from the body and weighed before and after removing the urine.

The data show that when a highly concentrated solution of urea was injected under the fetal skin the rate of secretion was accelerated (Group E). Although this is of interest in that it demonstrates the ability of the fetal kidney to secrete urine more rapidly than normally, the changes in osmosis thus produced suggest that the urea did not act solely as a specific diuretic agent. This view is supported by the data obtained from Groups F and G.

When the renal pedicles of the mother had been ligated for a period of 24 hours preceding tests,‡ it was found at autopsy that in most instances the bladder of the fetus was quite full (Group H). Ligation of the papilla for a period of 2 hours caused no significant change in the fullness (Group I).

To investigate whether the rate of secretion had been speeded up or whether some unknown factor had prevented the voiding of urine, 13 fetuses were subjected to laparotomy before tests and the urine was removed from

of such urine is being studied.

[1] Wells, L. J., *Anat. Rec.*, 1946, **94**, 504.

[2] Gersh, I., *Carnegie Inst. Wash., Contrib. to Embryol.*, 1937, **26**, 33.

[3] Wells, L. J., *Anat. Rec.*, 1946, **94**, 530.

‡ While this operation might raise the blood pressure of the mother, it does not necessarily follow that it would also raise the blood pressure of the fetus.

the bladder (Group J).§ At autopsy there were 4 cases in which the data suggest that the rate had been accelerated.‖ This sheds new light on Wolff's observation that in pregnant rabbits nephrectomy causes an increase in the amount of amniotic fluid.[4] Also, it raises again the question as to whether in human pregnancy an excess of amniotic fluid (hydramnios) may result from an abnormal accumulation of metabolites in the maternal blood stream, the metabolites acting in part by inducing an abnormally rapid formation of fetal urine.

§ In 3 additional fetuses in which the bladder was similarly visualized, squeezing it by means of blunt forceps forced its contents into the ureters and the renal pelves without causing any urine to emerge via the urethral outlet (action of sphincters?).

‖ In the other 9 cases, since the bladder was relatively empty, it is assumed that the hole made by puncturing the wall permitted urine to leak out.

[4] Wolff, B., *Arch. f. Gynäk.*, 1904, **71**, 224.

15452 P

Isolation of a Macromolecular Constituent with Properties of the Lansing Strain of Poliomyelitis Virus.*

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It has been reported from this laboratory that highly active preparations of the MV strain of poliomyelitis virus could be obtained by differential centrifugation of clarified extracts of infected medullae-cords of rhesus monkeys.[1] Application of the same procedures to brains and spinal cords of cotton rats infected with the Lansing strain resulted in appreciable quantities of high molecular materials which, however, showed only slight activity. Various modifications of the original procedure have been used in attempts to obtain highly active virus. It has been found that if the ether-clarified, aqueous extracts are frozen before they are subjected to ultracentrifugation, virus is obtained which is from 100 to 10,000 times as active on a nitrogen basis as the original clarified extract.

In the modified procedure the ether-clarified extract is prepared as previously described with the exception that filtration through celite is omitted. The slightly turbid solution is stored in 100 ml pyrex centrifuge bottles in a solid-CO_2 chest at from —35° to —60°C for at least 5 days and then allowed to thaw at room temperature. Before the ice present has completely melted, the solution is centrifuged in the cold room at 4°C in an angle centrifuge, and the supernatant liquid is removed; the sediment is extracted in the centrifuge twice with M/15 dipotassium hydrogen phosphate, and the latter extracts combined with the first supernatant liquid. The extracts obtained in this way are crystal clear, and depending on the length of time they have been frozen, contain from 75 to 51% of the nitrogen present before freezing. The appreciable amount of nitrogen precipitated is protein in nature, and as the specific activity of the extracts after prolonged freezing is the same or greater than before freezing, it appears that normal protein rather than virus is denatured during this treatment. The results of a typical experiment showing the effect of freezing for various lengths of time on the yield of soluble nitrogen and on specific activity are shown in Table I. Activity is expressed as the 50% infective dose ($I.D._{50}$) in young cotton rats and is calculated by the method of Reed and Muench.[2] The 50% infective dose is defined

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

[1] Loring, H. S., and Schwerdt, C. E., *J. Exp. Med.*, 1942, **75**, 395.

TABLE I.
Effect of Freezing on Soluble Nitrogen and Specific Activity of Extracts.

Exp No.	Days frozen	Soluble N. in extract	Specific activity*	
			Before freezing	After freezing
		%	g	g
73C	4	75	$10^{-6.5}$	$10^{-5.9}$
,,	45	53		$10^{-7.9}$
,,	65	51		$10^{-7.3}$
,,	120	53		$10^{-7.2}$

* Expressed as g nitrogen which produces poliomyelitis in 50% of young cotton rats as calculated by method of Reed and Muench.[2]

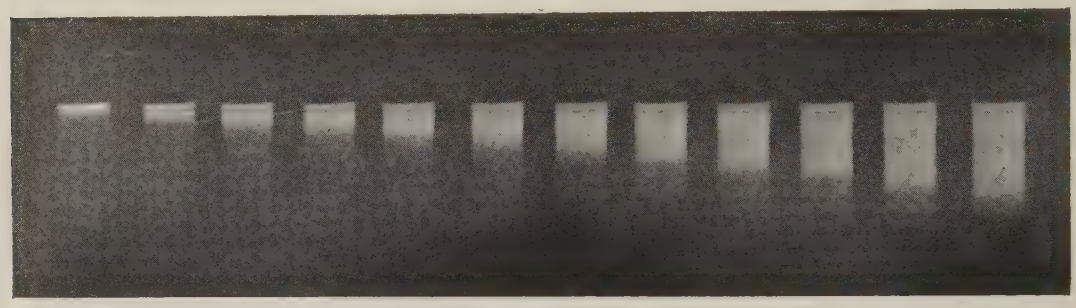

Fig. 1.
Sedimentation diagram of purified Lansing virus. (Speed 813 rps; interval between photographs, 2 min.; distance from center of rotor to top of cell, 8.04 mm; magnification of photograph, 5.07; temperature, 13.6°C).

as the amount of virus nitrogen which produces definite signs of poliomyelitis within 2 weeks in 50% of cotton rats from 4 to 6 weeks old injected intracerebrally with 0.05 ml of inoculum in 0.10 M acetate buffer at pH 4.

The purified virus is obtained by subjecting the clear extracts to 3 or 4 cycles of ultracentrifugal purification as previously described. When tested for specific activity, samples from different experiments have given values for $I.D._{50}$ of from $10^{-10.1}$ to $10^{-8.3}$ g of virus nitrogen. The purified material, which is present after the third ultracentrifugation as an amber-colored gel, dissolves in water to form opaque, slightly yellow-colored solutions, which have failed to show any evidence of streaming birefringence at concentrations where this phenomenon is readily detected with tobacco mosaic virus. Similarly, the sedimented gel is completely isotropic when viewed between crossed Nicol prisms in contrast to similar gels of tobacco mosaic virus which, as is well known, are highly birefringent. Solutions containing 0.2 mg of virus nitrogen per ml give a faint Millon's test for protein and a positive carbohydrate test with the orcinol-sulfuric acid reagent.[3]

The yield for third cycle virus has been from 0.1 to 0.2 mg of virus nitrogen[4] per 100 g of pooled brain and spinal cord. When the same purification procedures are applied to extracts of normal cotton rat brain and spinal cord, the percentage yields of macromolecular nitrogen after the extracts have been frozen for 5 and 10 days are of the order of 0.02 mg and 0.01 mg respectively. If the normal protein is present in the infectious extracts to the same extent it is found in normal tissue, it is evident that the maximum amount of normal macromolecular impurity present in the purified virus is of the order of 5 to 20%.

Examination of the virus samples for homogeneity in the McBain and Lewis 37 mm

[2] Reed, L. J., and Muench, H., *Am. J. Hygiene*, 1938, **27**, 493.

[3] Tillmans, T., and Phillipi, K., *Biochem. Z.*, 1929, **215**, 36.

[4] Tompkins, E. R., and Kirk, P. L., *J. Biol. Chem.*, 1942, **142**, 477.

transparent ultracentrifuge[5] have shown them to be relatively monodisperse as shown in Fig. 1 and to have a sedimentation rate of 83.5 ± 7.35 (average of 20 sedimentation runs on 5 preparations).

Conclusions. The results outlined above including (a) the presence in the purified samples of a uniform and high specific activity, (b) the demonstration that normal macromolecular constituents are largely eliminated by the purification procedures, and (c) the fact that the samples are relatively monodisperse in the transparent ultracentrifuge provide strong evidence that the preparations consist of essentially pure Lansing virus.

Presented at the Symposium of the Division of Biological Chemistry of the American Chemical Society on Biochemical and Biophysical Studies on Viruses at Atlantic City on April 12, 1946.

5 McBain, J. W., and Lewis, A. H., *J. Physical Chem.*, 1939, **43**, 1197.

15453 P

Electron Microscopy of Purified Lansing Virus.*

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Eleven different samples of purified Lansing virus prepared as described in the preceding paper have been examined in the Stanford election microscope.[1] The preparations were obtained from extracts that had been frozen from 4 to 26 days and gave specific activities, I.D.$_{50}$, from $10^{-10.1}$ to $10^{-8.3}$ g of virus nitrogen. Mounts were prepared by the usual technic of applying a small drop of dilute solution (10^{-8} to 10^{-7} g of virus nitrogen per ml) to the collodion film and allowing the solution to dry or by washing away the remainder of the drop with distilled water after 2 to 5 minutes. The mount was then either introduced directly in the microscope or it was treated by the gold shadowing technic of Williams and Wyckoff[2] before placing in the microscope.

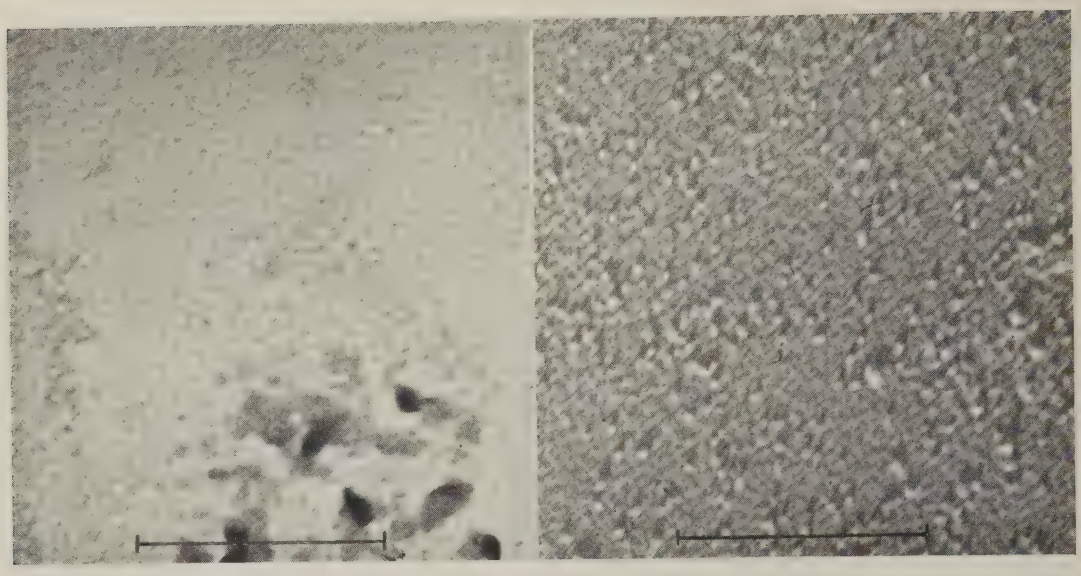
Although all the micrographs have not yielded uniform or conclusive results, they have in general shown the presence of spherical or possibly slightly asymmetrical particles ranging in size from 12 to 34 mμ. Examples of micrographs obtained when the virus was examined without gold shadowing as well as of those obtained by the gold-shadowing technic are shown in Fig. 1a and 1b. Fig. 1a was obtained from an extract that had been frozen for 26 days and purified by 2 cycles of ultracentrifugal purification and Fig. 1b from an extract frozen for 4 days and purified by 3 cycles of ultracentrifugal purification, the specific activities being I.D.$_{50}$ = $10^{-9.1}$ in both cases. Fig. 1a was obtained by the usual technic and Fig. 1b after gold shadowing.

In Fig. 1a there is some indication of slightly asymmetric particles with an average width of about 16 mμ and an average length of about 31 mμ or an average particle diameter of 23 mμ. In Fig. 1b this is not so apparent and the range of particle size as measured by shadow width is from about 15 to 34 mμ with an average value for particle diameter of 25 mμ. The slightly lower value for the non-shadowed specimen can probably be explained by the relatively low contrast between particles and background with the accompanying lack of definition in the particle image. Under these conditions the measured dimension is the greatest distance within the particle image of points of appreciable pho-

* Aided by grants from the National Foundation for Infantile Paralysis and the Rockefeller Foundation.

1 Marton, L., *J. Applied Physics*, 1945, **16**, 131.

2 Williams, R. C., and Wyckoff, R. W. G., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 265.



1a 1b

Fig. 1.

Electron micrographs of purified Lansing virus. The inscribed line in each case has a length corresponding to one micron. 1a. Concentration of 10^{-7} g virus nitrogen per ml applied to film. 1b. Same concentration as in 1a, mount placed in microscope after gold shadowing.

tographic contrast, which may not coincide with the true edges of the particle. In the case of the gold-shadowed specimens the shadow width is the measured dimension and because of the high degree of contrast this can be determined with considerably greater accuracy.

Of particular interest is the absence in all of the purified Lansing virus preparation of asymmetrical or thread-like particles as found by Gard[3] for both murine (Theiler's virus) and human poliomyelitis virus and as suggested by Bourdillon[4] for the SK mouse virus. While the results of Gard appear conclusive in the case of Theiler's virus, his conclusions with respect to human poliomyelitis were admittedly based on only a few experiments and open to other interpretations. The latter purified preparations from both infected tissues and feces contained components with sedimentation rates of the order of magnitude described in the preceding paper for the Lansing virus. Similarly while some filamentous particles were observed in his electron micrographs, there was also ample evidence for small, approximately spherical, particles. We feel, therefore, that Gard's results on human poliomyelitis are not necessarily incompatible with those presented here.

Conclusions. The results mentioned in the preceding paper as well as those given here lead to the conclusion that the Lansing virus, unlike Theiler's virus and probably the SK mouse virus, is a relatively spherical or slightly asymmetrical particle of about 25 mμ average particle diameter. It may be recalled that a somewhat similar conclusion was reported for the MV virus.[5] It appears to the present authors that such values for the size of these strains of poliomyelitis virus of human origin are also more in accord with the filtration end-point data and the conditions that have been found necessary for sedimentation in the ultracentrifuge.

Presented at the Symposium of the Division of Biological Chemistry of the American Chemical Society on Biochemical and Biophysical Studies on Viruses at Atlantic City on April 12, 1946.

[3] Gard, S., *Acta Med. Scand., Suppl.*, 1943, **143**, 173p.

[4] Bourdillon, J., *Arch. Biochem.*, 1943, **3**, 285.

[5] Loring, H. S., Schwerdt, C. E., and Marton, L., *Physical Rev.*, 1944, **65**, 354.

15454 P

Rôle of Bacteria in the Production of Tourniquet Shock in Rats.*

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Recently, Aub and coworkers[1] suggested that infection with *Cl. welchii* might be the cause of tourniquet shock. Ligation in dogs of the triceps surae muscles and then release after 5 hours, yielded toxic edema fluid contaminated with anaerobic organisms of the gas gangrene group. Intravenous injection of such fluid into normal dogs, in amount equivalent to that yielded by the donor dog, resulted in fatal shock in 9 out of 32 animals.

Prinzmetal *et al.*[2] produced shock in dogs by removal, crushing, and replacement of the quadriceps muscle. This was attributed to absorption of bacterial toxins from the crushed muscle. Shock was prevented by the local or systemic use of sulfamerazine.[3]

Method I. High unilateral rubber band tourniquets were used to interrupt the blood supply to one hind limb of 38 adult white male rats for 5 hours. Fatal shock developed after removal of constriction. The time of survival after tourniquet release ranged from 2 to 8 hours and averaged 3½ hours.

Immediately after death the injured limb and adjacent region were shaved, immersed about 5 minutes in 70% alcohol, and covered with tincture of iodine. Samples of muscle, each several mm in diameter, were then removed aseptically from the limb, placed in culture media and incubated at 37°C as follows: (a) anaerobically in Brewer's thioglycollate medium, (b) anaerobically in meat-infusion broth enriched with 0.5% dextrose, incubated in a Brewer anaerobic jar[4] utilizing an atmosphere of illuminating gas,[5] (c) aerobically in meat-infusion broth. The heart's blood was cultured in similar manner in all rats.

The tubes remained in the anaerobic jar for 4 days before removal. Bacterial stains were made after 4 days, 8 days, and before discarding at the end of 10 days.

II. Tourniquet shock was produced in rats pretreated with: (a) *sodium sulfadiazine,* 150 to 200 mg in 0.5 cc distilled water, intraperitoneally 30 minutes before removal of tourniquet; (b) *sodium salt of penicillin,* 1000 to 1500 units intramuscularly 6 hours before tourniquet release, and every 3 hours thereafter in one group of rats and every 2 hours in a second group; (c) *polyvalent gas gangrene antitoxin,* intramuscularly 6 hours before removal of tourniquet. Five or 10 times the amount of antitoxin proportional to the human dosage was given.

Fifteen rats were treated with sulfadiazine, 18 with penicillin, and 18 with antitoxin. Twenty untreated rats served as controls.

Results. I. Anaerobic cultures of muscle from the hind limb were negative in 29 of the 38 rats. Aerobic culture of muscle was negative in 34 animals. Anaerobic and aerobic cultures of the heart's blood were negative in 29 and 32 rats respectively. The positive anaerobic and aerobic cultures from both muscle and blood consisted of colon bacilli, non-hemolytic staphylococci, non-hemolytic streptococci, or diphtheroids. Most of these organisms appeared after 4 days of culture and were probably contaminants. *Cl. welchii* was not obtained in any instance.

In 10 control rats which received 1 cc of a 24-hour culture of *Cl. welchii* intramuscularly

* Aided by a grant from the Elisabeth Severance Prentiss Foundation.

1 Aub, J. C., Brues, A. M., Dubos, R., Kety, S. S., Nathanson, I. T., Pope, A., and Zamecnik, P. C., *War Med.*, 1944, **5**, 71.

2 Prinzmetal, M., Freed, S. C., and Kruger, H. E., *War Med.*, 1944, **5**, 74.

3 Freed, S. C., Kruger, H. E., and Prinzmetal, M., *Surgery*, 1944, **16**, 914.

4 Brewer, J. H., *J. Lab. and Clin. Med.*, 1939, **24**, 1190.

5 Brewer, J. H., and Brown, J. H., *J. Lab. and Clin. Med.*, 1938, **23**, 870.

TABLE I.
Results of Treatment.

Treatment	No. of rats	No. dead in shock	Mortality (%)	Time of survival (hr) range	Time of survival (hr) mean
Sulfadiazine	15	15	100	2-8	4
Penicillin	18	17	94	1-9	3¾
Gas gangrene antitoxin	18	17	94	2-9	4
None (controls)	20	19	95	2-13	4¾

in the hind limb just prior to tourniquet release, the organism was readily recovered after death by anaerobic culture of muscle.

II. Treatment with sulfadiazine, penicillin, or gas gangrene antitoxin had no appreciable effect on mortality or time of survival (Table I). The blood sulfadiazine levels of 7 rats at death ranged from 4 to 56.8 mg per 100 cc.

Discussion. The experiments of Aub and associates[1] involved open operative procedures in which bacterial contamination was possible, although organisms already present in the muscle may have been the source of infection. Shock was not produced in their donor animals but rather a state bordering on it. In the studies of Prinzmetal *et al.*[2] shock did not develop until 24 hours after replacement of muscle and death occurred after 2 or 3 days. This slowly developing form of shock due to bacteria is distinct from the acute form produced by tourniquet. In the latter, shock and death occur so rapidly as to make it unlikely that bacterial toxins are contributory.

Conclusion. In the experiments described in this article infection with *Cl. welchii* or other organisms did not prove to be a significant factor in the production of tourniquet shock in rats since:

(1) Anaerobic and aerobic cultures of muscle from the hind limb after death were usually sterile.

(2) Pre-treatment with sulfadiazine, penicillin, or gas gangrene antitoxin did not influence the development of shock, mortality or time of survival.

15455

Three New Salmonella Types: *S. richmond, S. daytona* and *S. tallahassee.**

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A. *S. richmond.* The sole representative of this type was isolated from the feces of a child affected with mild gastroenteritis. The isolation and preliminary examination of the culture was carried out by Mr. Forrest Spindle in the laboratories of the Virginia State Department of Health. Mr. Spindle later examined stool specimens of the remaining members of the child's family and investigated possible sources of infection. No carriers were found and the source of the infection was not determined.

The organism possessed the usual cultural and biochemical attributes of the Salmonella group. Glucose, arabinose, xylose, rhamnose, trehalose, mannitol, dulcitol, sorbitol and inositol were fermented with the production of acid and gas within 24 hours, while cel-

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TABLE I.
Flagellar Relationships of *S. tallahassee*.

	Serums									
	S. tallahassee				*S. cerro*			*S. düsseldorf*		
		Absorbed by				Absorbed by			Absorbed by	
Antigens	Unabsorbed	*S. cerro*	*S. düsseldorf*	*S. düsseldorf* and *S. cerro*	Unabsorbed	*S. düsseldorf*	*S. tallahassee*	Unabsorbed	*S. cerro*	*S. tallahassee*
S. cerro	10000	<100	500	<100	5000	2000	500	200	<100	<100
S. düsseldorf	10000	200	<100	<100	500	<100	<100	10000	2000	2000
S. tallahassee	20000	200	1000	200	1000	500	<100	1000	1000	<100

Figures indicate highest dilution at which agglutination occurred.

lobiose was fermented after 6 days incubation. Lactose, sucrose, raffinose and salicin were not attacked. H_2S was produced but indol was not formed nor was gelatin liquefied. Dextro-tartrate, levo-tartrate, mucate and citrate were utilized but meso-tartrate was not fermented.

Upon serological examination it was found that the culture was agglutinated to titre by *S. mikawashima* O serum (VI, VII) and in absorption tests removed all agglutinins from the serum. The organism was diphasic and phase 1 was agglutinated actively by, and removed all agglutinins from, serum derived from phase 1 of *S. mikawashima* (y). Phase 2 was agglutinated by serums prepared from all the non-specific phases of the genus. When tested with absorbed serums for factors 2, 3, 5, 6, 7, 10 and 11, it was agglutinated only by serums for factors 2 and 3. In absorption tests the culture removed all agglutinins from serum for phase 2 of *S. newport* (1,2,3...). Therefore, the antigenic formula of *S. richmond* is VI, VII: y—1,2,3... .

B. *S. daytona*. This type is represented by only one culture isolated from a stool specimen by Mrs. Mildred M. Galton in the laboratories of the Florida State Department of Health. The clinical condition of the individual from whom it was isolated is unknown. The biochemical characteristics of *S. daytona* differed from those given for *S. richmond* only in that rhamnose was not fermented during a 30-day period of observation and inositol and cellobiose were fermented respectively after 3 and 5 days incubation.

The culture was agglutinated to the titre of VI, VII serum and in absorption tests removed all agglutinins from that serum. *S. daytona* was diphasic and phase 1 was agglutinated by, and removed all agglutinins from, *S. thompson* (k) serum. Phase 2 was agglutinated by all non-specific serums. When tested with absorbed single factor serums, it was agglutinated only by serum for factor 6. In absorption tests with *S. anatum* (1,6...) serum it left a slight residue of agglutinins for the homologous strain but removed all agglutinins for phase 2 of *S. inverness* (1,6...) and phase 2 of *S. norwich* (1,6...). In regard to these results, it may be stated that the 1,6... phases of the genus, like the 1,5... phases[1,2] and the 1,7... phases[3] are not identical. The antigenic formula for *S. daytona* is VI, VII:k—1,6... .

C. *S. tallahassee*. This type is represented by 5 cultures, 3 of which were received from Mrs. Galton, while 2 were forwarded by Cmdr. L. A. Barnes of the National Naval Medical Center. One of the cultures from Florida was recovered from the feces of a case of gastro-enteritis, the remainder were

1 Salmonella Subcommittee, *J. Hyg.*, 1934, **34**, 333.

2 Edwards, P. R., Cherry, W. B., and Bruner, D. W., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 263.

3 Hormaeche, E., Peluffo, C. A., and Ricaud de Pereya, V., *J. Bact.*, 1944, **47**, 323.

isolated from the stools of normal human carriers. All of the cultures fermented glucose, arabinose, xylose, rhamnose, trehalose, dulcitol, mannitol, and sorbitol with the production of acid and gas within 24 hours. Four cultures fermented cellobiose after 7 to 12 days incubation. One culture fermented inositol promptly; the other 4 cultures did not attack this substance. Lactose, sucrose, salicin and raffinose were not fermented by any of the cultures. Three strains utilized *d*-tartrate; 2 did not. All the cultures utilized *l*-tartrate, *i*-tartrate, mucate and citrate. H_2S was produced but indol was not formed nor was gelatin liquefied.

The cultures were agglutinated to titre by O serum derived from *S. newport* (VI, VIII) and removed all agglutinins from the serum. They were monophasic and their H antigens were related to those of *S. cerro* (XVIII:z_4, z_{23}...) of Hormaeche and Peluffo[4] and *S. duesseldorf* (VI, VIII:z_4, z_{24}...) of Hohn.[5] The relationships of the H antigens of the 3 types are set forth in Table I. From the results it is evident that the 3 types have distinct antigens. It is further evident that factors z_{23} (*S. cerro* serum absorbed by *S. duesseldorf*) and z_{24} (*S. duesseldorf* serum absorbed by *S. cerro*) of Kauffmann[6] must now be redefined, since *S. tallahassee* is agglutinated by both. It is also necessary to assign a symbol (z_{32}) to the specific fraction of *S. tallahassee*. Therefore, z_{23} must now be prepared by the absorption of *S. cerro* serum with *S. tallahassee*, z_{24} by the absorption of *S. duesseldorf* serum with *S. tallahassee* and z_{32} by the absorption of *S. tallahassee* serum with *S. cerro* and *S. duesseldorf*. The diagnostic formula of *S. tallahassee* is VI, VIII:z_4, z_{32}. . . .

Summary. Three new Salmonella types were described: *S. richmond* (VI, VII: y—1,2,3...), *S. daytona* (VI, VII:k—1,6...) and *S. tallahassee* (VI, VIII:z_4, z_{32}...). Attention was directed to the necessity of redefining factors z_{23} and z_{24} since *S. tallahassee* was agglutinated by both serums as they previously were prepared.

[4] Hormaeche, E., and Peluffo, C. A., *Arch. Urug. de Med., Cir. y Espec.*, 1941, **19**, 125.

[5] Hohn, J., *Z. f. Hyg.*, 1936, **117**, 722.

[6] Kauffmann, F., *Die Bakteriologie der Salmonellagruppe*, Copenhagen, 1941.

15456

Flagella and Flagellar Antigens in "Non-Motile" *Salmonella* Cultures.*

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During the 25 years that antigenic analysis has been applied to the classification of *Salmonella*, it gradually has become an accepted view that flocculating, heat-labile (H) antigens were associated with flagella and motility. Conversely, nonmotile microorganisms are generally considered to be devoid of flagella and H antigens. However, occasional references to the presence of H antigens in nonmotile strains can be found. Kauffmann[1] described a nonmotile culture of *S. aberdeen* which flocculated well in i serum and which was used to prepare the diagnostic i serum distributed by the International Salmonella Center. Such findings naturally raise a question as to whether H antigens are

* The work reported here was in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director. It was supported in part by a research grant from the U. S. Public Health Service.

[1] Kauffmann, F., *Acta Path. et Microbiol. Scand.*, 1939, **16**, 278.

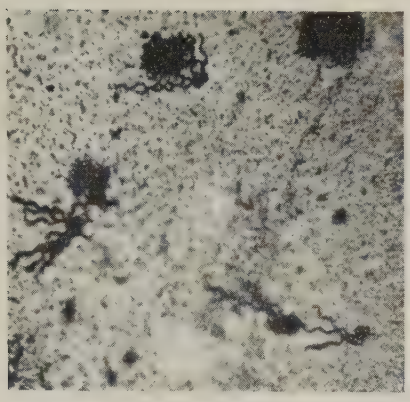

Fig. 1.
Flagella of *S. sandiego* strain. Van Ermengem's flagella stain. × 2000.

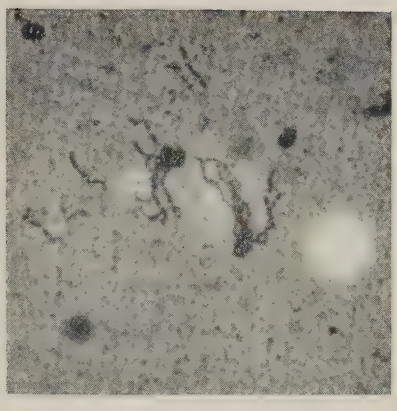

Fig. 2.
Flagella of III, XV:e,h strain. Van Ermengem's flagella stain. × 4000.

necessarily associated with flagella.

Several "nonmotile" *Salmonella* strains which flocculated with H serums were studied by the writers. One of these was a culture of *S. sandiego* received from Major R. H. Broh-Kahn. It was isolated and typed in the Central Diarrhea Control Laboratory of the Army Air Forces where it was recognized that although the culture possessed a normal complement of H antigens, it was nonmotile. Among nonmotile cultures typed in this laboratory, 3 had the formula VI, VIII:d and one was III, XV:e,h. The VI, VIII:d cultures were accidentally discarded but the *S. sandiego* and the III, XV:e,h strains were examined for flagella by the methods of Casares-Gil and of Van Ermengem. Cultures of actively motile bacteria, and of nonmotile bacteria which seemed to contain no H antigens were used for comparison. Large numbers of flagella were demonstrated in preparations from the motile cultures and from the nonmotile cultures which flocculated with H antigens. No flagella were observed in nonmotile cultures which failed to react with H serums. Photographs of flagella on the *S. sandiego* strain and the III, XV:e,h culture are illustrated respectively in Fig. 1 and 2. No differences were detected in the numbers or appearance of flagella present in the motile cultures and the flagellated nonmotile organisms.

The motility of the strains in question was judged by microscopic examination of broth cultures and by growth in stab cultures in the semisolid medium of Edwards and Bruner.[2] The VI, VIII:d type and the *S. sandiego* type exhibited no evidence of motility in serial transfers in semisolid medium over a period of several weeks. The III, XV:e,h bacteria began to migrate slightly from the site of inoculation after 3 serial transfers in semisolid medium, so that the growth had a rather beaded, fuzzy appearance. After 6 serial transfers it migrated slowly through the medium, producing an even turbidity. Behavior similar to that of the latter culture is not highly unusual in *Salmonella* cultures. However, such cultures usually do not flocculate with H serums until a fair degree of motility has been attained.

These observations constitute a confirmation of the results of Stuart and Wheeler[3] who, in their work on motility and swarming in paracolon cultures, noted flagella and H antigens on organisms which appeared nonmotile. They also noted that certain strains assumed motility after many months of growth in semisolid medium.

Two points are emphasized by the results presented here and by those of Stuart and Wheeler.[3] First, bacteria which appear nonmotile by accepted methods of examination may possess well-developed flagella and H antigens. This is added evidence of the

[2] Edwards, P. R., and Bruner, D. W., *Ky. Agric. Exp. Sta. Cir.* 54, 1942.

[3] Stuart, C. A., and Wheeler, K. M., 1946, personal communication.

flagellar nature of heat labile, flocculating antigens. As yet, no satisfactory explanation can be offered for the nonfunctional nature of the flagella in flagellated microorganisms which give no evidence of motility. Second, some degree of discretion should be exercised in characterizing a culture as being nonmotile. Unless a culture belongs to a well-known type which is known to lack motility, it should be examined carefully and be transferred serially in semisolid medium before it is concluded that motile forms cannot be obtained from it.

Summary. Two apparently nonmotile strains possessed well-developed flagella and flagellar antigens. One of the types (III, XV:e,h) eventually yielded motile elements after serial transfer in semisolid medium. The other *(S. sandiego)* gave no evidence of motility after similar treatment.

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Submerged Growth of Tubercle Bacilli from Pathologic Material in Dubos' Medium.

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The recent reports by Dubos[1] describing the remarkably rapid growth of Mycobacteria in a fluid medium containing purified phosphatides or certain synthetic nonionizing surface-active agents suggested the possibility that such media might be useful in the examination of pathologic material in the diagnostic laboratory.

During a period of 3 months 165 specimens were received for routine examination for tubercle bacilli by guinea pig inoculation. These specimens, untreated, were injected into guinea pigs according to the method in routine use. One hundred and forty-three of these specimens were examined by culture as well as guinea pig. For cultural examination, 5-20 ml volumes of the specimen were digested with an equal volume of 3% hydrochloric acid for periods varying from 15 minutes to 2 hours at room temperature, then neutralized to approximately pH 7.0 with 3% sodium hydroxide.[2] The digest was centrifuged and the sediment concentrated to 1/10th to 1/20th the original volume in sterile physiologic saline solution.

Several variations of basic media as described by Dubos[1] were used in this study (Table I). The various substances added to the basic media, together with the number of specimens examined in each are summarized in Table II. The specimens recorded as cultured in each basic medium were cultured in all the variations noted in Table II. Asolectin was first dispersed in a few ml of ether and added to the basic medium before autoclaving. Basic media were adjusted to pH 7.0-7.2 with normal sodium hydroxide and autoclaved at 15 lb. for 15 minutes. Glucose, albumin and Vegex were added to the autoclaved base in the form of sterile solutions. The media were dispensed in ordinary Pyrex test tubes 18-20 mm in diameter, stoppered with nonabsorbent cotton and sterilized in the autoclave. The various media were used in volumes ranging from 1 to 5 ml. Although growth can be obtained in as little as 1 ml of media, larger volumes (3-5 ml) were preferable since many cultures were kept in the incubator for 14-21 days, resulting in considerable reduction in volume by evaporation. Each lot of media was tested for growth-supporting capacity with stock strains of human (H37Va) and bovine tubercle bacilli and an acid-fast saprophyte *(M. phlei)* before

[1] Dubos, R. J., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 361.

[2] MacNabb, A. L., *Diagnostic Procedures and Reagents*, Am. Pub. Health Assn., 1941, Ed. 1, 281.

TABLE I.

Submerged Growth of Tubercle Bacilli from Pathologic Material: Basic Media.

Base		% in 1000 ml dist. water
1	Asparagine	.5
	Sodium citrate	.6
	Potassium phosphate (Dihydrogen)	.2
	Ammonium chloride	.1
	Magnesium sulphate	.05
	Ferric ammonium citrate	.005
2	Casein hydrolysate*	.2
	Sodium citrate	.15
	Potassium phosphate (Dihydrogen)	.1
	Magnesium sulphate	.06
	Sodium phosphate (Disodium)	.625
	Tween 80†	.05
3	N-Z Amine‡	.2
	Sodium citrate	.15
	Potassium phosphate (Dihydrogen)	.1
	Magnesium sulphate	.06
	Sodium phosphate (Disodium)	.625
	Mannitol	.5
	"Asolectin"§	.05

All minerals CP, not recrystallized.

* Product of Difco Lab., Inc., Detroit, Mich.

† A polyoxyethylene derivative of sorbitan monooleate, product of the Atlas Powder Co., Wilmington, Del.

‡ Product of Sheffield Farms Co., New York City.

§ Soya bean phosphatides, product of Associated Concentrates, Inc., Atlanta, Ga.

being used for culture.

Media were inoculated with 0.1-0.5 ml of the digested, concentrated specimen, these volumes representing from 2 to 10 times the volume of untreated sample as injected into the guinea pigs. In general the smaller volumes of inocula were used for sputa and necessarily, small volumes of specimen, while larger inocula were used for specimens such as urine or gastric aspiration. The inoculated media were incubated at 37°C and examined daily for one week for evidence of growth. Specimens were smeared as soon as visible growth appeared, in any case each was smeared after 2, 7, 14 and 21 days incubation. Smears prepared prior to the 21st day were made from the uncentrifuged culture, the final smears on the 21st day were made from the centrifuged sediment. Ziehl-Neelson or Kinyoun's modification[3] of the Ziehl-Neelson acid-fast stain was used.

Most of the positive specimens examined were obtained from cases in which tuberculosis was suspected clinically, but could not be established by direct microscopic examination of concentrated specimens. A few of the other positive specimens were obtained from cases in which tuberculosis was considered unlikely, the specimen having been submitted for exclusion.

As may be seen in Table II, there was excellent correlation between culture results and guinea pig inoculation, except with those specimens cultured in a medium which did not contain serum albumin. As Dubos observed,[4] the essential role of serum albumin may be merely a detoxifying action on inhibitory substances formed in the medium rather than nutritive; in either case it is evident that a small amount, either human or bovine, is necessary to insure growth. If the results obtained with the 4 specimens which were cultured in this medium are discarded, there remains a total of 29 specimens in the series which were positive either by culture or guinea pig or both. Twenty-six (90%) were positive by both culture and guinea pig. Two additional specimens were positive by culture alone (Table II), hence 28 or 96.6% of the specimens which contained tubercle bacilli were positive by culture. This figure does not differ significantly from the 27 positives (93.6%) obtained in guinea pigs; 26 positive by culture as well as by guinea pig; one, a bladder urine, positive by guinea pig alone. The 2 sputa which were positive by culture and negative by guinea pigs (Table II) were obtained from 2 different patients who previously had sputa or gastric aspirations positive both by culture and by guinea pig. A positive culture with a negative guinea pig on the same specimen can probably be accounted for by the difference in the volumes of inocula used in the culture and guinea pigs, respectively.

The kind and number of specimens with which positive results were obtained are summarized in Table III. The incubation range of from 5 to 27 days, with a mean of 11 days is in sharp contrast to the 8-12-week period

[3] Kinyoun, J. J., *Am. J. Pub. Health,* 1915, **5**, 867.

[4] Dubos, R. J., and Davis, B. D., *J. Exp. Med.,* 1946, **83**, 409.

TABLE II.
Submerged Growth of Tubercle Bacilli from Pathologic Material: Specimens Examined in Different Variations of Basic Media.

Medium	No. specimens examined	No. positive by culture	No. positive by guinea pigs
Basic 1 + 2% glycerin, 0.01% "Asolectin" 0.05% " 0.5% glucose, 0.01% " 0.05% "	12	0	4
Basic 1 + above variations, each with 0.2% (human) serum albumin*	45	12	12†‡
Basic 1 + 2% glycerin, 0.05% "Asolectin" 0.5% glucose, 0.05% "Asolectin" each with 0.2% (human) serum albumin	28	7	6†
Basic 2 + 0.5% glucose, 0.2% Vegex,§ 0.2% Bovine Fraction V‖	36	6	6
Basic 3 + 0.2% Vegex, 0.3% Bovine Fraction V	22	3	3
Total	143	28	31

* By courtesy of the Mass. Dept. of Public Health.
† Two sputa negative in guinea pigs, positive in cultures. In both instances previous specimens on these 2 patients were positive in guinea pigs and culture.
‡ One bladder urine positive in one guinea pig, negative in culture.
§ Product of Vegex Corp., New York City.
‖ Product of Armour Lab., Chicago, Ill.

TABLE III.
Submerged Growth of Tubercle Bacilli from Pathologic Material: Incubation Time of 28 Positive Specimens.

Material cultured	No. specimens	Incubation in days Range	Mean
Sputum	10	5-21	8.3
Gastric aspiration	6	7-27	12.1
Urine*	7	7-20	12.7
Miscellaneous			
Joint fluid	2		
Pus	1		
Autopsy material†	1	6-21	10.6
Epididymis	1		
Total	28	5-27	11.1

* Five bladder, 2 catheterized specimens.
† Tuberculous meningitis. Antemortem and postmortem spinal fluids, pus from arachnoid, periaortic node, uterus and tube all positive. Another case with an antemortem diagnosis (clinical) of tuberculous meningitis was negative by culture. However, *H. capsulatum* was isolated later from an antemortem spinal fluid.

usually required for diagnosis by routine guinea pig inoculation. Twenty-three (82%) of the positive results were obtained in 14 days or less. The 5 specimens requiring incubation periods of more than 14 days (Table IV) were of the kind which usually contain extremely small numbers of tubercle bacilli. The incubation periods observed in this experiment probably represent the maximum since after the seventh day the culture as a rule was not resmeared until the 14th and 21st days. If daily examination by smear, or more frequent examination of the centrifuged sediment were practicable on all specimens, a still further reduction in time might be expected.

TABLE IV.
Submerged Growth of Tubercle Bacilli from Pathologic Material: Positive Specimens Requiring More than 14 Days Incubation.

Material cultured	Incubation in days
Gastric aspiration	18
Bladder urine	20
Sputum*	21
Joint fluid	21
Gastric aspiration	27

* Guinea pigs negative. Previous specimens on same patient positive by guinea pigs and by culture.

The amount of growth obtained with different specimens showed considerable variation, but could not be correlated with differences between the various lots of media recorded in Table II. It seemed that the amount of growth depended more on the nature of the specimen (number of tubercle bacilli in the inoculum?) or perhaps strain differences than on the particular medium used. Some cultures presented massive "rafts" of tubercle bacilli many immersion fields in area, while others required diligent search of the smears before a few small clumps of tubercle bacilli could be demonstrated. In either case the arrangement of cells in clumps connoted reproduction. In all cases, further incubation or transfer of a slowly growing culture to fresh media resulted in a more profuse growth. The morphology of tubercle bacilli grown in these media seemed to be slightly different from that observed on the various coagulated egg media. The individual cells tended to be longer and slightly thicker. Beaded and banded forms, such as are seen in smears made directly from tuberculous lesions, with bipolar staining or large, nonacid-fast granules are common.

Discussion. This preliminary study suggests that with reasonable care in their use, these new synthetic media can be invaluable in the laboratory diagnosis of tuberculosis. The cultural results reported here perhaps are not directly comparable to the results in guinea pigs with regard to sensitivity, since concentrated specimens were compared in culture with the unconcentrated specimens in guinea pigs. Concentrated inocula were used in order to determine whether or not the media would support the growth of tubercle bacilli from pathologic material without attempting a direct comparison of the inocula volume for volume in guinea pigs and cultures.

Digestion for as short a time as 15 minutes at room temperature with 3% hydrochloric acid satisfactorily eliminated concomitant bacteria from specimens such as urine, gastric aspiration and joint fluids which were not grossly contaminated. Shorter periods of digestion were preferable, in view of the possibility of sterilizing a specimen which contained only a few tubercle bacilli. Sulfadiazine, 30 mg %, was added to the media in which certain of the specimens were inoculated without acid digestion. Although a few positive results were obtained in such media, the method seemed of limited value since the Gram negative microorganisms were the most troublesome contaminants in these media. Also, the drug may crystallize on the slide, making staining and examination more difficult.

These media will support the growth of a variety of nonacid-fast microorganisms, such as *B. subtilis*, some strains of *E. coli* and an unidentified aerobic, Gram negative spore-bearing rod. This latter microorganism, a common dust contaminant, not only withstands the usual acid digestion but also produces an abundance of short, narrow, free spores which retain the carbolfuchsin and might conceivably be mistaken for tubercle bacilli. Certain strains of yeast, frequently present in sputum and gastric aspiration also withstand acid digestion and grow profusely in these media. However, the growth of tubercle bacilli has been observed, even in a badly contaminated culture.

As is the case with other preparations, these media also support the growth of acid-fast saprophytes,[1] hence with the present extent of knowledge of the metabolic differences among this group of microorganisms, they cannot be used alone for the specific bacteriologic identification of tubercle bacilli. However, in those instances where specific bacteriologic identification of the acid-fast organism is indicated, the injection of a pure culture of acid-fast organisms, rather than material taken directly from the patient, into guinea

pigs should result in a more rapid diagnosis. It might be noted that the strain of *M. phlei* mentioned earlier produced a yellow-orange pigment in these media, in contrast to the white, nearly colorless growth produced by the human and bovine strains of tubercle bacilli.

Conclusion. The new synthetic media recently described by Dubos for the rapid cultivation of Mycobacteria can be successfully employed to isolate tubercle bacilli from various pathologic material. A combination of rapid culture with guinea pig confirmation where indicated should result in a marked reduction of the time required for the laboratory diagnosis of tuberculosis.

15458

Significance of Thromboplastic Activity of Antigens Used in Complement-Fixation Tests.

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Most antigens employed in serologic tests, particularly crude preparations, cause nonspecific reactions in varying degree. They are also usually anticomplementary. The underlying cause of these effects has not been understood. In practice, antigens are diluted to avoid the anticomplementary reaction as far as possible and sera are either used in amounts beyond the range where nonspecific reactions occur or the specificity is evaluated on the basis of the reacting dilution. In some instances nonspecific reactions have been avoided by heating sera above their inactivation temperature, to 60° or 65°C.[1,2] In general this is considered poor serologic practice because of the destructive effect of higher temperatures upon antibodies and of the reactivating effect of such heated sera upon the hemolytic activity of complement.

Relationships between the coagulative and complement activity of serum have been previously noted.[3,4] Cephalin, which is highly thromboplastic, inhibits complement to a degree closely parallel to its clotting activity. This thromboplastic activity of cephalin may be markedly enhanced by the addition of normal mammalian sera, even after inactivation at 56°C for 30 minutes.[5] Since many antigens possess thromboplastic qualities it seems likely that their inhibitory action on complement may be due to the enhancing effect of inactivated serum upon these thromboplastic qualities. It has been our experience that reduction in the thromboplastic quality of the tissue extract used in the complement-fixation test for syphilis diminished the number of nonspecific reactions obtained. The purified antigen, cardiolipin, now widely used, does not possess thromboplastic activity.[6]

The interdependence of these phenomena may have practical importance in the serologic study of sera in neoplastic and virus diseases in which tissue extracts are frequently used as antigens. Normal rabbit sera and fresh tissue extracts have been found to fix complement, which led to the conclusion that normal tissue antibodies were present in such animals.[7] Since fresh tissue extracts are highly

1 Casals, J., and Palacios, R., *Science*, 1941, **93**, 162; *J. Exp. Med.*, 1941, **74**, 409.

2 Thomas, L., and others, PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 121.

3 Wadsworth, Augustus, Maltaner, Frank, and Maltaner, Elizabeth, *J. Immunol.*, 1936, **30**, 417.

4 Wadsworth, Augustus, Maltaner, Frank, and Maltaner, Elizabeth, *J. Immunol.*, 1937, **33**, 297.

5 Maltaner, Frank, and Maltaner, Elizabeth, *Arch. Biochem.*, 1943, **2**, 37.

6 Maltaner, Frank. Unpublished data: Report, June 8, 1942.

7 Kidd, J. G., and Friedewald, W. F., ***J. Exp. Med.***, 1942, **76**, 543.

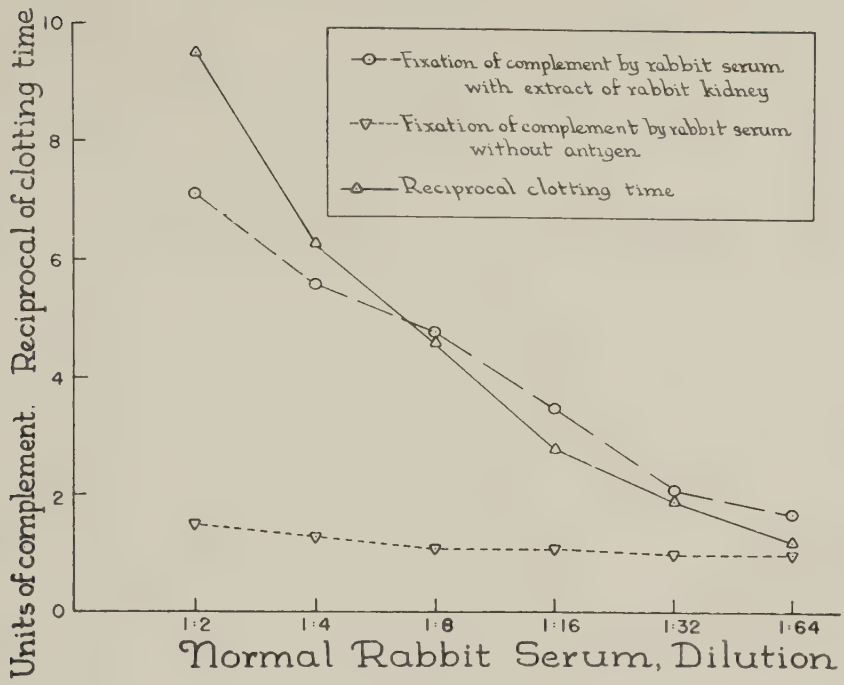

Fig. 1.
Comparative tests of the complement-fixing and clotting activities of normal rabbit sera inactivated at 56°C for 30 minutes and fresh extracts of rabbit kidney.

thromboplastic, a comparative study was made of the enhancement of their thromboplastic and complement-fixing activities by normal rabbit sera; also of the effect upon both activities of heating the normal sera beyond their reactivation temperature.

Saline extracts of liver and kidney of normal rabbits and guinea pigs were prepared as described by Kidd and Friedewald[7] and used as antigens. The complement-fixation tests were conducted in 0.3 ml volumes. Complement was titrated against a 5% suspension of sheep cells maximally sensitized with an equal amount of amboceptor to determine the unit (the amount required for 50% hemolysis). Dilutions were prepared so that the required number of units, 1—2 in the control tests of serum or antigen alone and 2, 3 and 6 units in the tests of serum and antigen, were contained in 0.1 ml.[8] Fixation was for 2 hours at room temperature. Sensitized cells were added in amounts of 0.2 ml and 15 minutes in the water bath at 37°C was allowed for hemolysis. No effort was made to determine the optimum dosage required for maximum reaction because of the labile character of the tissue extracts.

The clotting activity of the inactivated sera was determined by testing comparable dilutions of serum and antigen with recalcified oxalated rabbit plasma. Plasma was secured by the paraffin technic[4] and the amount of calcified saline used was sufficient to clot the plasma in 20 to 30 minutes in the presence of the test dose of tissue extract. The test was conducted in the water bath at 37°C and the interval between adding plasma and definite clot formation was measured.

Fig. 1 shows that parallelism existed between the units of complement fixed and the reciprocal of the clotting time when various dilutions of normal rabbit serum were tested with rabbit kidney extracts. Similar results were secured when guinea-pig kidney extracts and liver extracts of both guinea pig and rabbit were tested with several different rabbit sera. When sera were inactivated at 56°C,

8 Wadsworth, A. B., *Standard Methods of the Division of Laboratories and Research of the New York State Department of Health*, 2d ed., Baltimore, Williams and Wilkins, 1939, p. 243.

60°C, and 65°C respectively the diminution in complement fixation was closely paralleled by the decrease in clotting activity.

Conclusions. These results give further evidence of the correlation between clotting and complement activity and indicate that antigens possessing thromboplastic activity may give nonspecific complement fixation.

15459

A Note on the Relationship Between the Intermedius and Minimus Types of *C. diphtheriae.**

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Frobisher[1] and Eller and Frobisher[2] described bacteriologically and epidemiologically an outbreak of diphtheria in Baltimore and called attention to 2 hitherto undescribed types of diphtheria bacilli. One of these is a virulent saccharose-fermenting variety. The other, with which the present communication deals, is characterized by the following special properties: (a) the production, on the cystine-tellurite agar, used routinely in these laboratories and previously described,[3] of very small, often hardly visible colonies that are always flat, but which may be either rough or smooth; (b) failure to ferment dextrose promptly (*i.e.*, within 48 hours at 37°C), *when first isolated.* In the article referred to,[1] attention was also called to the fact that other small-colony strains of diphtheria bacilli were encountered during the epidemic described but that the term *minimus* type was reserved for the minute-colony strains which failed to ferment dextrose as do strains ordinarily encountered in Baltimore and tested under the same conditions.

It is well known that small-colony types of *C. diphtheriae* are not uncommon. Such forms have been described by Corbett and Phillips,[4] Morton,[5] and Mittag.[6] However, failure to ferment dextrose by such forms has not been described previously and it seemed desirable to designate so distinctive a variety by a special name, following the example of Anderson *et al.*[7] Since publishing this description several workers, in personal communications, have suggested that the *minimus* type is in reality identical with the *intermedius* type described by Anderson *et al.* It is the purpose of this note further to clarify the status of the *minimus* type among other small-colony types.

First, as to colony form, the original description of the *intermedius*-type colony states that the colonies are small as are those of the *mitis* type. In other respects the organisms of the *intermedius* type are closely similar to the *mitis* type except that in broth cultures the *intermedius* type produces a granular sediment, rather than a smooth turbidity like the typical *mitis* strains. In this respect the *intermedius* strains are also like the *minimus* strains. No pictures of the *intermedius* colonies were shown in the original article,[7] but colored plates were presented which illustrate the *gravis* and *mitis* colonies. In a monograph by Dudley[8] a similar colorplate shows *intermedius* colonies and these are not at all like colonies of the *minimus* type.

* Aided by a grant from the International Health Division of The Rockefeller Foundation.

1 Frobisher, M., Jr., Adams, M. L., and Kuhns, W. J., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 330.

2 Eller, C. Howe, and Frobisher, M., Jr., *Am. J. Hyg.*, 1945, **42**, 179.

3 Frobisher, M., Jr., *J. Inf. Dis.*, 1937, **60**, 99.

4 Corbett, L., and Phillips, G. C., *J. Path. Bact.*, 1897, **4**, 193.

5 Morton, H. E., *Am. J. Path.*, 1932, **8**, 605.

6 Mittag, G., *Zentr. Bakt. Parasitenk.*, 1937, I Orig., **138**, 426.

7 Anderson, J. S., Happold, F. C., McLeod, J. W., and Thompson, J. G., *J. Path. and Biol.*, 1931, **34**, 667.

8 Dudley, S. F., May, P. M., and O'Flynn, J. A., *Active Immunization Against Diphtheria*, His Majesty's Stationery Office, 1934.

Second, no mention was made by Anderson *et al.*[7] of unusual fermentative properties of the *intermedius* type as originally described. It was stated by those workers that the fermentation reactions of the *intermedius* type were normal, *i.e., mitis*-like. This is definitely unlike the *minimus* type. It would appear then, that *intermedius* and *minimus* strains are 2 distinct varieties of small-colony types of diphtheria bacilli.

In order to clarify still further the difference between the *intermedius* and *minimus* types several strains of each were cultivated side by side on the same Petri plates and in tubes of dextrose broth all made from the same lot of medium and inoculated at the same time. It was our good fortune to be able to make such a comparison due to the kindness of Dr. J. W. McLeod, of the University of Leeds, in sending us some time ago (1936), a collection of *gravis, mitis,* and *intermedius* strains. The growth on the slants as originally received from England was suspended at that time in a few drops of defibrinated rabbit's blood and immediately desiccated. They remained undisturbed for 10 years. This technic and its value have recently been described by Frobisher, Pai and Hakim.[9] In February 1946, the 10 *intermedius* strains from Leeds were taken from the vacuum jar, transferred to blood-dextrose broth to revive them and, when growth had developed (24 hours at 37°), each of 10 plates of cystine-tellurite agar was streaked on one-half of its surface with one of the 10 cultures. The other half of each plate was inoculated in exactly the same way with one of 5 *minimus* strains which had been desiccated in exactly the same manner as the English strains, but for only a few months. Simultaneously, tubes of heart-infusion broth containing 1% dextrose were respectively inoculated with one drop of a broth culture of each of the 15 cultures being compared.

After the usual period of incubation (48 hours at 37°) the growth on the plates was examined. On 4 of the plates there was no difference between the *minimus* cultures and those labeled *intermedius*. On the other 6 plates the *intermedius* colonies were larger, more raised, darker, smoother and more glistening, *i.e.,* clearly more *mitis*-like than the *minimus* colonies. A differentiation between the 2 types of growth could easily be made with the unaided eye. However, both would certainly be classed as dwarf- or small-colony variants.

The point of particular interest was that, on examining the dextrose broth the *minimus* cultures and the 4 Leeds cultures the colonies of which were of the *minimus* type, were found to have remained alkaline. No fermentation occurred during 7 days of incubation. The 6 cultures from Leeds the colonies of which were more *mitis*-like, had produced marked acidity in 48 hours. It was clear that of the 10 Leeds strains, all were definitely of the small-colony variety, but 6 were of the type known as *intermedius,* whereas the other 4 were of the type we have designated *minimus.*

The question of growth in the broth tubes requires some discussion. The *minimus* strains, whether from Leeds or Baltimore, do not grow as luxuriantly on any media so far tested, as do the *mitis, gravis, indeterminate,* or *intermedius* strains. This is doubtless a reflection of their small-colony formation on solid media. However, they grow sufficiently in broth so that one would reasonably expect dextrose to be fermented were the organisms capable of doing so. After several subcultures on artificial media they acidify dextrose broth and then grow more vigorously, but they still grow as sparsely on media not containing dextrose. Their failure to acidify dextrose broth immediately after isolation is in contrast with other strains; a distinctive feature, whatever its explanation.

Summary. A comparative study was made of *intermedius*-type strains of *C. diphtheriae* obtained from Dr. J. W. McLeod, Leeds, England and *minimus*-type strains described by Frobisher in Baltimore. The *minimus* type was found to be a distinct colony variant and was differentiated from strains described as *intermedius* by Anderson, *et al.* Other small-colony variants have also been found differing from both the *intermedius* and *minimus* types.

9 Frobisher, M., Jr., Pai, S. E., and Hakim, S., in preparation.

15460

Isolation of Virus of Lymphogranuloma Venereum from Blood and Spinal Fluid of a Human Being.*

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Although the virus of lymphogranuloma venereum has been isolated frequently from inguinal buboes, genital and rectal lesions, there have been few successful isolations of this agent from other parts of the body.[1] Sabin and Aring[2] unequivocally demonstrated the virus in the spinal fluid of a patient with meningoencephalitis. Von Haam and D'Aunoy[3] also reported its isolation from the spinal fluids of 2 patients suffering from lymphogranuloma venereum, but identification of their agents is open to some question, since it was based principally on positive skin reactions with infected mouse brain in patients with clinical lymphogranuloma.

The agent of lymphogranuloma venereum has been found in the blood of infected experimental animals,[1] but it has not previously been reported in the blood of a human being. The present communication reports the isolation of this virus from the blood and the spinal fluid of 1 of 8 proved cases of the disease.

Methods. The technic of isolation and identification of the virus of lymphogranuloma venereum used in this laboratory is given in detail elsewhere.[4] The salient features are as follows: Fresh material is inoculated intracerebrally in 0.03 cc amounts into each of 6 young adult Swiss mice, and in 1 cc amounts into the yolk sac of 6 6-day-old fertile eggs. Mice usually show signs of illness on the first passage, whereas blind passages frequently have to be made with yolk sac material before infection becomes evident. The virus is established in each host by a number of successive passages of infected material. Elementary bodies are readily found in spreads of infected mouse brains and yolk sacs stained by the Macchiavello method.

Further identification of these agents as the virus of lymphogranuloma venereum is based on tissue tropisms and sulfonamide inhibition. Of the various agents of the psittacosis-lymphogranuloma group, only lymphogranuloma venereum has the 2 characteristics of infectivity for mice by the intracerebral route of inoculation and of susceptibility to sulfonamide inhibition.[5]

Results. The 8 patients concerned in this report were young colored adults, 7 males and one female, and were all studied in the early stage of lymphogranuloma venereum infection. Four were tested within the first 4 days, one on the sixth day, one on the 14th day and 2 on about the 20th day of disease. Systemic manifestations in these patients were no more pronounced than in other patients with this disease. Furthermore, none of them showed evidence of meningeal involvement, and spinal fluid examinations in all 8 cases gave normal cell counts and protein levels.

The virus of lymphogranuloma venereum was isolated from bubo pus from every one of the 8 patients. Blood was examined for the presence of virus in 6 of them, and similar studies were carried out on the spinal fluid

* This work was aided by a grant from the Venereal Disease Division of the U. S. Public Health Service.

[1] Literature summarized by: Stannus, H. S., *A Sixth Venereal Disease*, Baillière, Tindall, and Cox, London, 1933, Ch. 11; van Rooyan, C. E., and Rhodes, A. J., *Virus Diseases of Man*, Oxford University Press, London, 1940, Ch. 16; Koteen, H., *Medicine*, 1945, **24**, 1.

[2] Sabin, A. B., and Aring, C. D., *J. A. M. A.*, 1942, **120**, 1376.

[3] von Haam, E., and D'Aunoy, R., *J. A. M. A.*, 1936, **106**, 1642.

[4] Wall, M. J., *J. Immunol.*, in press.

[5] Hamre, D., and Rake, G., *J. Infect. Dis.*, 1944, **74**, 206.

of 5 patients, at the time when virus was obtained from the buboes. The virus of lymphogranuloma venereum was isolated from both blood and spinal fluid of one patient (No. 161), a 20-year-old woman, tested on the sixth day of the disease. The agents from her blood and spinal fluid produced signs of infection in chick embryos on the first passage, whereas material from her bubo required one blind passage before infection was evident. From 8 to 17 passages were made on all 3 agents. Elementary bodies were demonstrated in spreads of infected mouse brains and yolk sacs, and the tissue tropisms and susceptibility to sulfonamide therapy, characteristic of the virus of lymphogranuloma venereum, were found to be properties of these agents. In addition, serologic identification of the viruses was established by neutralization of their toxins by hyperimmune serum prepared in chickens against a known strain (J.H.) of the virus of lymphogranuloma.[4,6,7]

Summary. Eight patients with acute lymphogranuloma venereum, proved by isolation of the virus from buboes, were examined for the presence of virus in blood or spinal fluid, or both. The virus was found in the blood and also in the spinal fluid of one patient. The clinical features in this case were not significantly different from those of the other 7 cases. This is the first reported instance of isolation of the agent of lymphogranuloma venereum from the blood of a human being.

6 Rake, G., and Jones, H. P., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 86; *J. Exp. Med.*, 1944, **79**, 463.

7 Hilleman, M. R., *J. Infect. Dis.*, 1945, **76**, 96.

15461

Effect of Diet on the Response of Chicks to Folic Acid.*

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Previous studies[1] in our laboratory indicated that chicks showed less growth, feather development and hemoglobin production when preparations of impure folic acid (obtained from Texas University) and the third *L. casei* factor (obtained from Lederle Laboratories, Inc.) were added to our basal ration containing sulfasuxidine than when added to the basal ration alone. Scott *et al.*[2] found the third *L. casei* factor to be somewhat active in the presence of sulfasuxidine, particularly in the presence of the pyridoxic acids. Subsequent work[3] showed that the activity of synthetic folic acid is apparently decreased when it is fed in diets containing sulfasuxidine. In an attempt to determine the function of folic acid by measuring its activity under different dietary conditions it was found that changes in diet may markedly alter the response observed when a given amount of folic acid is fed to chicks.

Experimental. Day-old White Leghorn chicks weighing between 40 and 50 g were divided uniformly into groups of 10, placed in electrically-heated cages with raised screen bottoms and fed the diets described in Table I. It is important to note that the level of vitamins was considerably higher than that normally used. The largest and smallest

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1 Briggs, G. M., Jr., Luckey, T. D., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1945, **158**, 303.

2 Scott, M. L., Norris, L. C., Heuser, G. F., and Bruce, W. F., *J. Biol. Chem.*, 1945, **158**, 291.

3 Luckey, T. D., Moore, P. R., Elvehjem, C. A., and Hart, E. B., *Science*, 1946, **103**, 682.

TABLE I.
Composition of Basal Diets.

Constituent	494	504	505*	506	507	508	509	510	512
Dextrin	61	39	59					46	
Sucrose				61					46
Glucose					61				
Starch						61			
Corn meal							67		
Casein†	18	50		18	18	18	12	18	18
Egg albumin			30						
Gelatin	10			10	10	10	10	10	10
Cystine	.3			.3	.3	.3	.3	.3	.3
Salts	6	6	6	6	6	6	6	6	6
Soybean oil	5	5	5	5	5	5	5	20	20
Folic acid content/100 g‡	4	6	44	1	2	5	17	4	—

Vitamins per 100 g: A, 2400 I.U.; D_3, 240 I.U.; E, 0.6 mg; K, 0.1 mg; thiamine Cl, 0.6 mg; riboflavin, 1.2 mg; Ca pantothenate, 4 mg; choline Cl, 300 mg; nicotinic acid, 10 mg; pyridoxine Cl, 0.8 mg; biotin, 0.04 mg; and inositol, 200 mg.

* 200 μg of biotin per 100 g diet were added to counteract the avidin present in this diet.

† Crude casein was extracted by stirring 3 times with 1½ volumes of 95% alcohol for a total of 6 hours at 78°C.

‡ As measured with *S. faecalis*[5] after acid hydrolysis.[6]

chicks in each group were discarded at the end of the first and at the end of the second weeks (with the one exception noted) and the experiment was terminated when the remaining 6 chicks were 4 weeks old. The methods used for determining hemoglobin values and measuring changes in feather development were the same as those used previously.

Results. Effect of Protein. The data in Fig. 1A show that good hemoglobin and growth responses and normal feather development were obtained when 50 μg of folic acid per 100 g of ration was added to the regular dextrin diet. When the casein in the diet was increased to 50% (Fig. 1B), a low level of folic acid produced a striking response in growth and hematopoiesis, but the best growth obtained with this diet did not approach that obtained with the lower level of casein. With the high casein diet the feather development was not normal even when high levels of folic acid were fed. When the protein of the ration was replaced with 30% commercial egg albumin (Fig. 1C), growth was poor and the folic acid did not improve the rate of growth or hemoglobin production. This level of egg albumin supplied 40 μg of folic acid per 100 g of basal ration and this intake was undoubtedly sufficient to give the response observed without any added folic acid. The fact that growth with this diet was better than that obtained with the high level of casein and the fact that no dermatitis was observed indicate that the biotin added was adequate to counteract the avidin supplied by the egg albumin.

Effect of Carbohydrate. When sucrose was substituted for dextrin (Fig. 1D), the folic acid supplements produced little response until a level of 50 μg was used and maximum responses were not obtained until 100 μg were added. These results are in marked contrast to those obtained when dextrin was the carbohydrate. When glucose was substituted for dextrin (Fig. 1E), 20 μg of folic acid per 100 g of ration produced a growth response but allowed little feather development. Normal feathers were not obtained until 100 μg of folic acid was fed. The maximum growth attained with glucose as well as with sucrose was considerably less than that obtained with dextrin. Feather development was also irregular when low levels of folic acid were fed in the diet containing starch (Fig. 1F). The feathers of chicks receiving 10 μg of folic acid were less developed than those of chicks receiving no folic acid although the growth was 30 g better in the former chicks.

[5] Luckey, T. D., Briggs, G. M., Jr., and Elvehjem, C. A., *J. Biol. Chem.*, 1944, **152**, 157.

[6] Luckey, T. D., Briggs, G. M., Jr., Moore, P. R., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1945, **161**, 395.

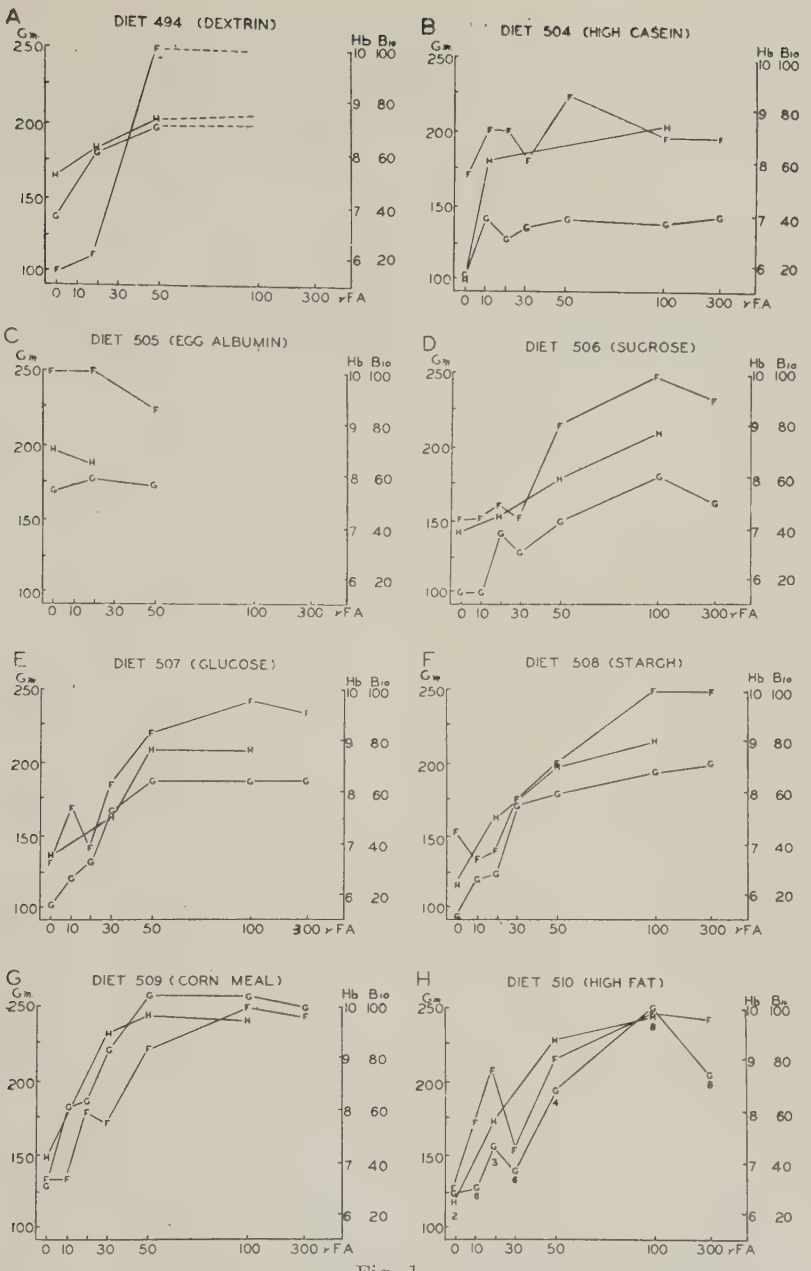

Fig. 1.

The responses in growth, feather development, and hematopoiesis observed in 4-week-old chicks fed different basal diets and 0-300 μg of folic acid per 100 g of diet.

Gm = Average weight of the 6 chicks in each group at 4 weeks and is indicated by the letter G.

Hb = Average grams of hemoglobin per 100 cc of blood for 4 chicks in each group and is indicated with the letter H.

B_{10} = Average feather development and regression for each group of chicks and is indicated with the letter F. O = very poor feathers and 100 = very good.

The numbers in Fig. 1H indicate the number of chicks out of 8 that survived on that diet. Chicks on diets other than No. 510 died only in those groups receiving no folic acid. The number dead for each basal group is: No. 494—0, No. 504—2, No. 505—0, No. 506—3, No. 507—3, No. 508—2, No. 509—0.

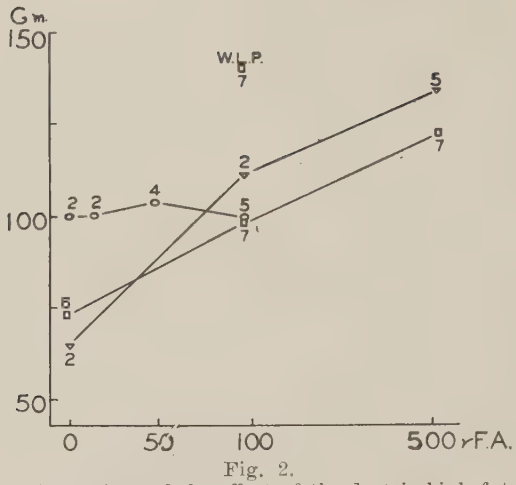

Fig. 2.

Comparison of the effect of the dextrin-high fat, sucrose-high fat, and sucrose-normal fat diets upon the response of chicks at 3 weeks to different amounts of folic acid and liver.

○ = Diet No. 510 (dextrin-high fat).
△ = Diet No. 512 (sucrose-high fat).
□ = Diet No. 506 (sucrose-normal fat).

The average weight of each group of 7 chicks is plotted against the γ of folic acid per 100 g of diet. The number of chicks surviving 3 weeks is given for each group. The group designated W.L.P. was fed Diet No. 506 + 5% whole liver powder (≅ 100 γ folic acid) + 100 γ folic acid.

When cornmeal was fed at 69% of the diet (Fig. 1G), 10 μg of folic acid per 100 g of diet produced a good growth response but had little effect on the feather development. When 50 μg of folic acid was added feather development still was not as complete as on the dextrin diet. The hemoglobin values obtained with this diet were higher than those obtained with the other diets.

Effect of Fat. When 20% soy bean oil was included in the basal diet containing dextrin as the carbohydrate (Fig. 1H), a marked feather response to low levels of folic acid was noted while only small growth responses were obtained with levels of folic acid which had previously been adequate. Thus a comparison of the basal group with the group receiving 10 μg of folic acid per 100 g of diet shows essentially no difference in weight but a definite difference in feather development. Evidently under this regimen folic acid fulfilled the requirement for vitamin B_{10} but not for vitamin B_{11}. The growth and hemoglobin values obtained in this series were higher than those obtained when a soluble carbohydrate was fed. Since several chicks died during the second week no chicks were discarded at the end of that week, so the results for this series are based upon 8 chicks per group. From the mortality data indicated in the figure it is evident that high levels of folic acid were effective in counteracting this condition.

In a second experiment, in which 7 chicks weighing 38-42 g were started in each group, the results at the end of 3 weeks indicated again (Fig. 2) that folic acid reduced the high mortality observed on the dextrin, high-fat diet. A growth response with added folic acid was not observed. When sucrose was used as the carbohydrate (diet No. 506) the results indicated that extremely high levels of folic acid might be required to produce maximum growth while 5% of whole liver powder in the diet produced a growth response which could not be accounted for by its folic acid content. This effect has also been observed with a dextrin diet.[3] Whether it is due to a known vitamin, amino acid or a new factor has not been determined. When the sucrose high-fat diet (No. 512) was fed, 500 μg of folic acid per 100 g of diet did not completely counteract the mortality. All chicks receiving the high-fat diets had an extensive dermatitis similar to that reported by Ben-Ami Ben-Dor,[4] but this does not seem to be a lethal dermatitis under the conditions of our experiments.

Discussion. When low levels of folic acid were used in purified diets, there appeared to be no direct relationship between the growth response and the feather development. With high-casein diets no correlation was observed between feather development and the amount of folic acid in the diet. More than 30 μg of folic acid per 100 g of diet was needed to produce a feather response with the sucrose diet, while a marked feather response with low levels of folic acid may be seen when dextrin is fed with either 5 or 20% fat. Growth responses with low levels of folic acid were good on diets containing dextrin, starch, glucose, and corn meal (except the high fat-dextrin diet) and poor with the sucrose and

[4] Ben-Dor, B., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 495.

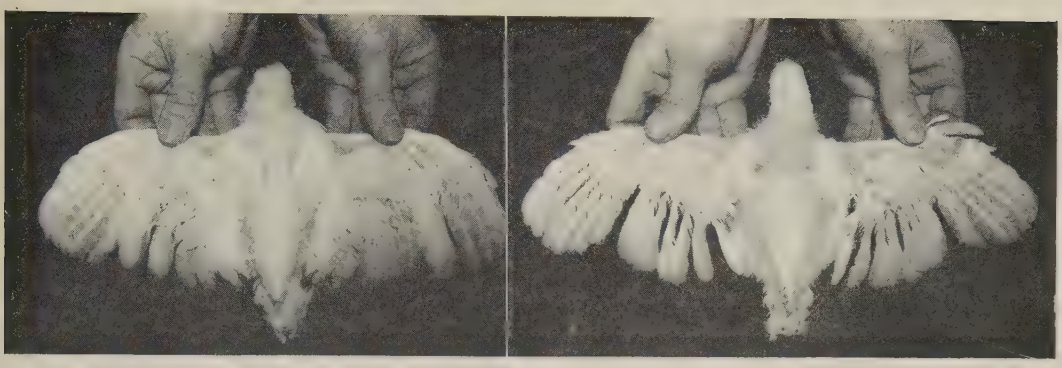

Fig. 3.

Typical results showing differences in the feather development in chicks growing at approximately the same rate. The data for the groups of chicks represented by 2 typical chicks above are:

No. 494 (dextrin)	Diet	No. 509 (corn meal)
50	γ folic acid per 100 g diet	30
100	feather development (B_{10})	55
200 g	weight	220 g

high-fat diets.

Lack of correlation between feather development and rate of growth was observed on the high-casein, glucose, starch and corn-meal diets when the folic acid content was low. Although the differences are not large, they are significant in view of the fact that variation was minimized by starting with chicks of uniform weight which were hatched together and raised at the same time in one set of batteries. The uniformity was improved by discarding 40% of the large and small chicks early in the experiment. A typical difference in feathering is given in Fig. 3. The chick on the left, which received the dextrin diet + 50 μg of folic acid per 100 g of diet, weighed somewhat less and feathered much better than the chick on the right which was raised for 4 weeks on the corn-meal diet + 30 μg of folic acid per 100 g of diet. This lack of correlation indicates the existence of 2 compounds fulfilling these functions. It is interesting to note that most of the work with vitamins B_{10} and B_{11} was carried out with low levels of folic acid (3-25 μg per 100 g of diet).

These differences may be due: to variations in the utilization of the added folic acid by chicks in the different groups; to variations in possible reactions of the folic acid with the other nutrients or metabolites; or to an indirect action of folic acid on the intestinal flora whereby the production of vitamin B_{10} (the feather factor) or vitamin B_{11} (the growth factor) would be increased. It seems very unlikely that one compound could give these different responses unless it caused the production or affected the utilization of more than one nutrient.

The great variation in response obtained from a given level of folic acid under different conditions indicates that no definite requirement for this compound can be made without specifying the type of diet. Thus although 10 μg of folic acid per 100 g of diet gave maximum growth when fed with a high-protein diet, maximum growth was not attained with a diet containing sucrose as the carbohydrate or 20% fat until the level of folic acid was 100 μg per 100 g of diet. Whether this is evidence for the indirect action of folic acid or an indication of the biological function of this compound needs further study.

In general 3 types of responses seem to be obtained with these different diets (Fig. 4). The anemia is the most severe with the starch or high-casein diets and is the most readily counteracted with small amounts of folic acid. The other extreme is seen with the diets containing sucrose or glucose as the carbohydrate. In these cases the anemia is not severe and the response to folic acid is slow. An intermediate response to low levels of folic acid

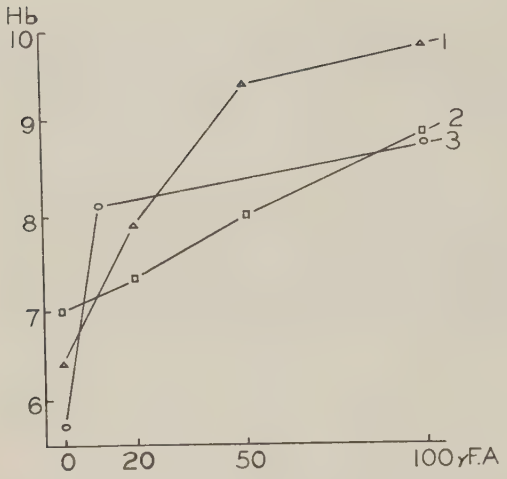

Fig. 4.

Summary of Hemoglobin Production.

The hemoglobin values (in grams per 100 cc of blood) are plotted against the μg of folic acid per 100 g of diet.

1 = High-fat diet (the curves obtained with the corn meal diet or the regular dextrin diet are similar).

2 = Sucrose diet (this is similar to the curve obtained with glucose as the carbohydrate).

3 = High-casein diet (the starch diet produced a similar curve).

is obtained with the corn-meal or dextrin (high or low fat) diets but higher levels of folic acid produce more hemoglobin than is obtained with the other diets. Since low levels of folic acid are effective when fed with high-protein diets and ineffective in diets containing high fat, glucose or sucrose, it seems evident that treatment of this type of anemia should be facilitated by high-protein, low-fat, and low-soluble carbohydrate diets.

The reason for the superior growth and hemoglobin production with a corn-meal diet is not known, but it is evident that corn is, when properly supplemented, a good dietary constituent.

Summary. A study of the effects of dietary constituents upon the activity of synthetic folic acid in chick nutrition shows that no definite requirement for this compound can be established since the response to a given amount of folic acid depends upon the type of ration used. Folic acid produced the least response with high-fat diets or diets containing glucose, sucrose, or starch as the sole carbohydrate, and the best response with diets containing high protein, low fat, or corn-meal and dextrin as the carbohydrates.

Hemoglobin responses were better on diets containing dextrin or corn-meal than diets containing other carbohydrates.

Feather development cannot be correlated with either the amount of folic acid in the diet or the rate of growth of the chicks when low levels of folic acid are included in the diet.

Whole liver substance when added to a diet containing sucrose as the carbohydrate gave an increased growth response that could not be attributed to its folic acid content.

We thank Merck and Company, Inc., Rahway, N.J., for the crystalline vitamins, Wilson Laboratories, Chicago, Ill., for gelatin and whole liver powder, Allied Mills, Peoria, Ill., for soy bean oil, and Lederle Laboratories, Inc., Pearl River, N.Y., for the folic acid.

15462

Effect of Some of the B Complex Vitamins upon Chick Tissue Cultures.

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In view of the prominence which some of the vitamins of the B Complex are assuming in the role of therapy, especially in some disorders resulting in alterations of the skin and of the nervous system, it was thought of interest to discover whether or not some

of these vitamins had any specific effect upon chick tissues grown *in vitro*.

In the first series of experiments nicotinamide, pyridoxine (B_6), thiamin chloride (B_1), pantothenic acid (as calcium pantothenate), and riboflavin were chosen for use. Skin and brain as ectodermal derivatives and heart as of mesodermal origin were selected from 10-day-chick embryos as the test tissues. Chicken plasma and Ringer-Tyrode solution in equal parts constituted the growth medium for all control cultures. The experimental cultures were planted in equal parts of fresh chicken plasma and Ringer-Tyrode solution to which had been added the various vitamins so that for any one of the above vitamins the series of final dilutions in the cultures were 2000; 1000; 500; 250; 125 and on occasions 62.5 γ per cc of medium.

In the second series of experiments folic acid and biotin* were added to the Ringer-Tyrode solution before the same tissues from 12-day embryos were planted. The cultures were planted similarly to those in the preceding series except that lyophiled plasma[1] was substituted for fresh and that the concentrations of vitamins were very much less. The biotin groups were set up in a concentration series of 5, 2.5, 1.25, 0.625, 0.312, 0.156 γ per cc of medium; the folic acid groups in 3.6, 1.8, 0.9, 0.45, 0.225, 0.112 γ per cc of medium, since assays of these vitamins in chick embryo tissue of 12 days incubation are rather low.[2] Sterilization of all the vitamin solutions was accomplished by passing them through a Seitz filter. They were made up fresh at frequent intervals to avoid the possibility of deterioration.

The cultures were all made by the hanging drop method using as far as possible, bits of tissue of comparable size in any one planting. Sterile precautions were used throughout and all cultures were incubated at 37.5°C. The pH of the experimental cultures at the time of planting varied from 7.4-7.6. There were 1369 experimental cultures and 412 controls—one set of controls for approximately each 3 sets of experimental cultures from a single embryo planting. When all experimental cultures in several successive dilutions of a given vitamin showed lack of growth or of any form of migration of cells they were not repeated. If migration and growth were nearly equal to or slightly better than those of the controls, this experiment was repeated once to several times to be sure that the effect might not be due to the idiosyncrasy of a particular planting. Microscopic observations were made daily upon the cultures, but no effort was made to keep planimetric records of growth since cultures of brain tissue particularly, do not yield themselves to this form of observation. As far as could be determined by careful contrasting observations between all control and experimental cultures, no alterations or abnormalities in appearance of the various cell types could be detected in the vitamin cultures.

The outgrowth from both control and experimental cultures consisted of: heart—mostly fibroblasts, macrophages, occasional groups of endothelial- or mesothelial-like cells and very slight or no increase of muscle tissue; brain—myriads of axones from within the explant, some neurones, microglia (macrophages) and cells interpreted as astrocytes from their staining reactions; and skin—many fibroblasts, some capillary sprouts, scanty epithelial sheets, macrophages and a few melanophores.

In all these experiments embryo juice was not employed as a growth stimulant since this is obtained from steeping all the minced tissues of 10-12-day embryos in Ringer-Tyrode solution. The supernatant fluid must contain apart from other cell substances a complement of vitamins. Embryo tissues as sug-

* The author wishes to express his appreciation to Merck & Co., Inc., for supplying all the vitamins used in the first series of experiments and also for their extensive bibliographies relating to them. Thanks are also due to Dr. Philip Handler of the Department of Biochemistry for the use of a folic acid concentrate (approx. 60% pure substance, Lederle) and crystalline biotin (Merck); to Dr. Wm. A. Perlzweig for advice and criticism; and to Jane Stanley Craig for her technical assistance.

1 Hetherington, Duncan C., and Craig, Jane Stanley, Proc. Soc. Exp. Biol. and Med., 1939, **42**, 831.

2 Williams, Roger J., Taylor, Alfred, and Cheldelin, Vernon, *Univ. of Texas Publ. No.* 4137, 61-66.

gested by the available data on the content of liver, brain, and heart[3] are good sources—particularly the liver—of all the B vitamins in question. The total vitamin content of the liver declines approximately 50% by hatching time even though nicotinic acid is synthesized by the developing chick.[4] To what extent the chicken plasma of the medium itself might have contained B vitamins is not positively known but it was assumed to be a negligible factor throughout the experiments.

Since the growth stimulating effect of embryo juice was absent, it must be kept in mind that all these cultures grew probably by virtue of their own stored growth energy or growth substances and/or perhaps, to a certain extent, by utilizing the plasma and also cell autolysates from within the explant. As a consequence their existence was shortened and degeneration set in usually after the third or fourth day in culture although some lived for more than 12 days.

The literature relating specifically to growth of cultures with vitamins is practically nonexistent. The effects of these substances upon any organ or tissue, derived principally from deficiency states produced within the whole living animal, and later treated by addition of the missing vitamin or vitamins, must needs be evaluated by different standards from effects of these same vitamins upon tissue cultures. In these a mere fragment of tissue is living in an abnormal environment removed from all the modifying factors of the whole living animal. The control cultures might be considered as bits of tissue living in a multiple vitamin deficiency state since the only vitamins present are essentially those carried into the culture by the bit of tissue itself. The addition then of a given vitamin to these cultures should, if alteration of growth occurs, be considered perhaps as evidence of a specific effect for that vitamin within the limits of the experiment.

TABLE I.

Showing Maximal Concentrations of B Vitamins in γ per cc of Medium for Equivalent or Better Growth of Brain, Heart and Skin in Tissue Cultures from Chick Embryos of 10-12 Days Incubation.

Vitamin	Tissues explanted		
	Brain	Heart	Skin
Riboflavin	62.50	62.50	62.50
Nicotinamide	125.00	125.00	250.00
Thiamin	500.00	*	125.00
Pyridoxine	250.00	62.50	125.00
Calcium pantothenate	125.00	500.00	250.00
Biotin	5.00	1.25	0.62
Folic acid	1.50	1.50	3.00

* Growth rate of cardiac cultures was unaffected by any of the concentrations used.

In the first series of experiments, concentrations of the vitamins of 2000 and 1000 γ per cc of medium were definitely detrimental to the growth of the skin and brain and with the exception of thiamin, to the heart also. Migration of cells did not take place and the explants rapidly became opaque and disintegrated—a sign of death. Until growth became nearly equal to or better than that of the controls the vitamins were considered to be toxic or in some instances suppressive.

The heart cultures consisting principally of fibroblasts were wholly unaffected in growth rate by thiamin in all dilutions used. This is in accord with the work of Hengstman[5] using the same vitamin on cultures of chick heart and also that of Paterson and Thompson[6] employing pure cultures of chick embryo fibroblasts with aneurin chloride hydrochloride (thiamin) even in greater concentrations than in the present experiment.

In the second series of experiments with folic acid and biotin none of the concentrations tested was suppressive for any of the tissues and in general growth was a little better throughout than with the vitamins of the first series.

The maximal concentrations for equivalent or better growth of all the experimental cultures appear in Table I. It must be stated here that the differential between growth "equal to" and "better than" was so slight

3 Taylor, Alfred, Mitchell, Herschel K., and Pollock, Maxwell, A., *Univ. of Texas Publ. No.* 4137, 67-80.

4 Dann, W. J., and Handler, Philip, *J. B. C.*, 1941, **140**, 935.

5 Hengstman, Von H., *Z. g. Vitamin Forschng.*, 1938-39, **8**, 208.

6 Paterson, Edith, and Thompson, Mary V., *Biochem. J.*, 1943, **37**, 501.

that the general conclusion must be drawn from the experiments that, riboflavin, nicotinamide, thiamin chloride, pyridoxine, calcium pantothenate, folic acid and biotin produced a negligible effect upon growth; that is, proliferation of cells was not enhanced.

A much more interesting observation noted was the effect of the B vitamins upon *survival* of the outgrowths. Many cultures, regardless of the rate of proliferation, outlived their controls for some time after all growth was static. In other words, they did not degenerate as rapidly even though active growth had ceased. This was most noticeable in brain cultures with biotin and folic acid in which the controls were badly degenerated after 5 days whereas the vitamin cultures, especially those containing folic acid, survived an additional 7 days.

It is common knowledge among tissue culturists that to maintain growth and multiplication of cells in culture, one must supply a medium containing some stimulating substance, the best of which is embryo tissue extract.[7] Cultures grown in media without embryo extract grow only to a limited extent and degenerate rapidly as compared with cultures of the same tissues grown with embryo juice. Since the B vitamins investigated here in certain concentrations produced equivalent growth in cultures of brain, skin, and heart where embryo juice was not employed it seems probable that they may, in part at least, be responsible for supplying some of the growth factors of tissue extracts.

It is interesting that data from the Texas group of investigators[8] on the analysis of beef heart and beef heart nuclei for all of the B vitamins indicate that the content of nuclei for each vitamin was 3 to 4 fold greater than for the cytoplasm except for pyridoxine which was slightly less and biotin which was about half as much. If such analyses hold true for other tissues in general it might be suggested that, with the exception of biotin and pyridoxine, the B vitamins might be essential for maintaining the integrity of the nuclei; biotin and pyridoxine, for cytoplasmic effects. Since the nucleus is considered paramount for the maintenance of the cell as a living unit, might the longevity noted in some of the vitamin cultures over their controls be due to an effect principally upon the nuclei?

Summary. Cultures of skin and brain (ectodermal derivatives) and heart (mesodermal derivative) in the presence of the B complex vitamins in certain concentrations, grew as well as, or slightly better than their own controls without vitamins. From the results of all the experiments the conclusion must be drawn that the added B complex vitamins have a negligible growth stimulating effect upon tissue cultures of skin, brain and heart. However, they did seem to affect favorably the longevity of the cultures, particularly those of brain, with biotin or folic acid. These outlived the majority of their controls in good (biotin) and excellent (folic acid) condition by a span of days more than once again as long.

[7] Ebeling, A. H., *J. Exp. Med.*, 1913, **17**, 273.

[8] Isbell, Edith R., Mitchel, Herschel K., Taylor, Alfred, and Williams, Roger, *Univ. of Texas Publ. No.* 4237, 81-83.

15463 P

Production of Atheromatosis in the Aorta of the Chicken by Administration of Diethylstilbestrol.*

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The experimental production of a hyperlipemic atheromatosis by methods other than the feeding of cholesterol has not hitherto been reported. It is shown here that atheromatosis can be induced in the bird by the subcutaneous implantation of diethyl-

* Aided by grants from the Christine Breon Fund for Medical Research.

Fig. 1.
Thoracic aorta of Bird 1548. Sudan IV × 120.

stilbestrol, a procedure that results in a sustained hyperlipemia.[1] This provides a new and simple procedure for the study of experimental atheromatosis. It has the distinct advantage in that aortic lesions so produced more closely resemble the spontaneous lesions in birds than do those produced by the exogenous administration of cholesterol.

Pellets of diethylstilbestrol, each weighing approximately 25 mg, were implanted into White Leghorn cockerels obtained from a single hatch. A single pellet was injected into each bird when it was 3.3 months old and at the following intervals thereafter: 30 days, 40 days, and 65 days. Blood samples were taken for lipid analyses at monthly intervals during the course of the experiment. All birds used in this study were fed a stock diet, the composition of which has been described elsewhere.[2] The birds were sacrificed by exsanguination and their tissues examined histologically.

Thoracic Aorta. The thoracic aortas of 5 of the 7 birds that were examined from 6 to 7 months after implanation of the first pellet contained lesions that were grossly visible. These were lemon yellow in color and appeared as elliptical plaques that measured from 1 to 2 mm in width and from 12 to 15 mm in length. In 4 of the birds these areas were slightly elevated. They were most numerous on the convex side of the aortic arch, although in one bird they were also found in the brachiocephalic arteries and in the lower thoracic and abdominal portions of the aorta.

Microscopic lesions of the aortic arch were found in all 7 of the diethylstilbestrol-injected birds. These consisted of zones of intimal thickening elevating the endothelium; when extensive enough, this process produced the visible plaques (Fig. 1). The thickened intima was composed of large vesicular fibroblastic cells supported by reticulum and

1 Entenman, C., Lorenz, F. W., and Chaikoff, I. L., *J. Biol. Chem.*, 1940, **134**, 495.

2 Lorenz, F. W., Chaikoff, I. L., and Entenman, C., *J. Biol. Chem.*, 1938, **123**, 577.

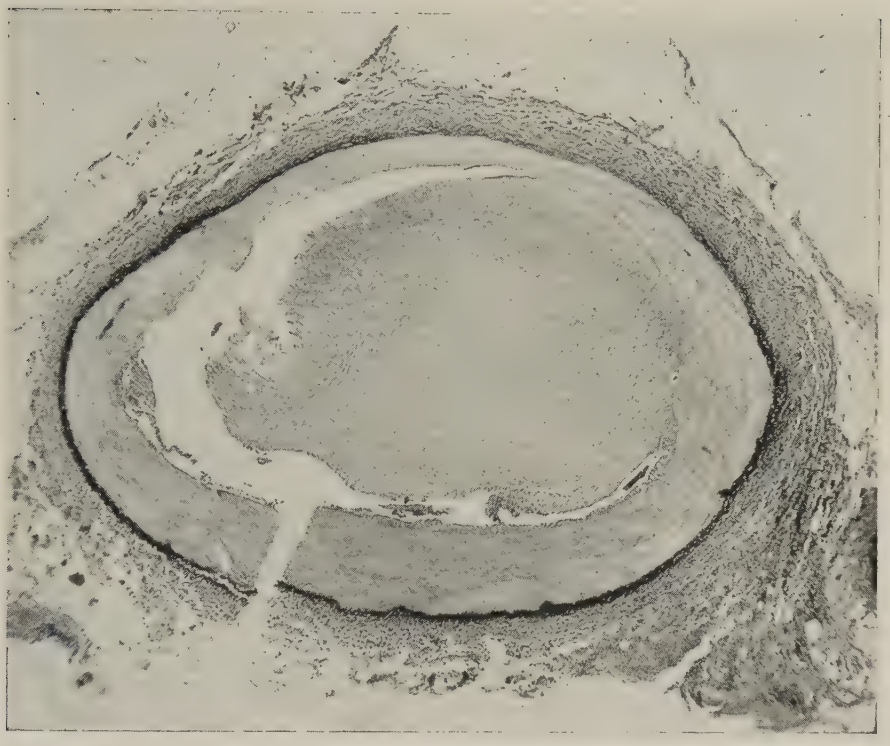

Fig. 2.
Abdominal aorta of Bird 1565. Weigert-Van Giesen × 36.

collagenous fibers that lay perpendicular to the endothelial surface. A few foam cells of the macrophage type were found in these intimal plaques. The intimal thickening was produced not only by an infiltration of lipids but also by an increase in the number of connective tissue cells. The cytoplasm of the connective tissue cells of the inner portion of the media and of those parts of the intima that were not thickened was also foamy and vesicular.

By the use of Sudan IV and Nile blue sulfate stains it was demonstrated that the cytoplasm of all connective tissue cells and macrophages in the intima and of all connective tissue cells of the adjacent media of the aortic arch was distended by small lipid droplets (Fig. 1). The bulk of this material was not cholesterol, although moderate numbers of refractile crystals (cholesterol) were visible with polarized light.

Nine control birds were sacrificed at 11 months of age. In 8 no gross or microscopic alterations were found in the thoracic aorta. In a single bird a negligible number of minute lipid droplets were observed in a few of the connective tissue cells of the intima and adjacent media in the arch of the aorta.

Abdominal Aorta. Eight of the 9 control birds that were sacrificed at the age of 11 months showed gross or microscopic evidence of the "spontaneous" lesion of the abdominal aorta that has been described for the domestic fowl.[3] In 4 of the birds there were longitudinal, elongated ridge-like plaques on the anterior wall just above the bifurcation. They measured 1-2 mm in width and 7-15 mm in length and were pearly grey in color. Microscopic examination showed that they consisted of dense hyalinized connective tissue fibers lying parallel to the circumference of the vessel and enclosing vesicular fibroblasts. These cells in the deeper and central portion of the plaques were filled with lipid droplets. Small amounts of lipid material were also

[3] Dauber, D. V., *Arch. Path.*, 1944, **38**, 46.

present in the adjacent media.

Although the "spontaneous" lesions were also found in the stilbestrol-injected birds, they differed from those found in the controls in that they contained an abundant amount of lipid material some of which was cholesterol. Moreover, degenerative changes as well as calcification had occurred in the plaques of the stilbestrol-treated birds (Fig. 2).

In none of the control birds did the level of total lipids of plasma exceed 500 mg per 100 cc. Although considerable fluctuations occurred in the lipid level of the plasma of the stilbestrol-treated birds, values for total lipids well over 10,000 mg per 100 cc were commonly found; in a single bird the concentration of total lipid rose to 17,800 mg. Neutral fat accounted for most of the rise in total lipid. In the control birds the levels of total cholesterol in plasma did not exceed 130 mg, whereas in the injected group values above 500 mg were frequently observed.

15464 P

A Transitory Decrease in Glucose Tolerance Following Experimental Lesions in the Central Nervous System.*

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In the process of determining daily postoperative blood sugars in animals with major traumatic experimental lesions in the brain stem, it was noted that an immediate postoperative hyperglycemia seldom occurred,[1,2] but in some instances a definite hyperglycemia appeared after the onset of postoperative feeding, *i.e.*, when blood sugar was determined 18 to 24 hours after the previous meal.

It seemed apparent that this postfeeding hyperglycemia was due to the presence of a decrease in the animal's tolerance for carbohydrates, the 18- to 24-hour hyperglycemia being the direct result of an excessive prolongation of the meal tolerance curve. For the purpose of ascertaining if this were true, the glucose tolerance in dogs has been followed for extended periods after major intracranial procedures.

Methods. The intracranial procedures consisted of (1) hemisection and transection of the midbrain and pons, (2) destruction of the ventral tuberal portion of the hypothalamus, and (3) removal of the cerebellar vermis and the cerebellum in its entirety. Operative procedures were carried out under sodium pentobarbital anesthesia (30 mg per kg of body weight), and have been described in detail elsewhere.[3-5]

The glucose meal consisted of 2 g per kg of body weight, administered in a 20% aqueous solution by stomach tube. Tests were run 24 hours after the previous meal, and postoperative feeding was begun the day after operation, *i.e.*, 48 hours after the last preoperative meal. Blood sugar was determined by the Gibson modification of the Folin-Wu method.[6]

Results. Elevated and prolonged tolerance curves, ranging from hardly detectable (see Dog 3-C, Fig. 1) to very striking effects (see Dogs 5 and 4-C, Fig. 1) were routinely encountered following these procedures. This

* Aided by a grant from the John and Mary R. Markle Foundation.

1 Donhoffer, C., and MacLeod, J. J. R., *Proc. Roy. Soc., Series B*, 1932, **110**, 125.

2 Bell, D. J., Horne, E. A., and Magee, H. E., *J. Phys.*, 1933, **78**, 196.

3 Keller, A. D., Roy, R. S., and Chase, W. P., *Am. J. Physiol.*, 1937, **118**, 720.

4 Keller, A. D., *J. Neurophysiol.*, 1945, **8**, 275.

5 Keller, A. D., and Hamilton, J. W., Jr., *Arch. Surg.*, 1938, **37**, 760.

6 Gibson, R. B., PROC. SOC. EXP. BIOL. AND MED., 1929, **27**, 480.

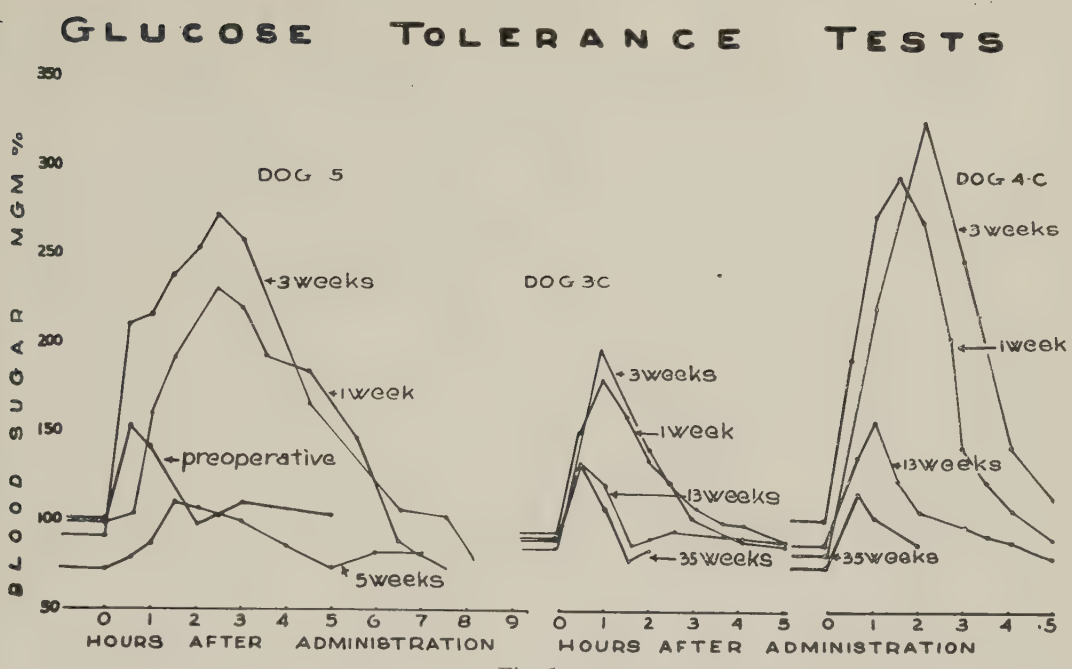

Fig. 1.

The operation in Dog 5 consisted in a double transection of the brain stem, one through the caudal extent of the diencephalon, the other through the pons. The operation in Dog 4C was a radical removal of the cerebellar vermis. The operation for Dog 3C was a radical removal of the entire cerebellum.

decrease in tolerance increased progressively for some time after operation, rapidly at first, then more slowly. After a maximum was reached, it was maintained for some time, after which it receded, ultimately returning to normal (see all animals, Fig. 1).

There was no certain indication that a lesion in any one part of the central nervous system was more prone to precipitate striking effects.

Discussion. The similarity of this phenomenon to starvation diabetes is at once apparent. The possibility that dietary factors may have enhanced the height and duration of the early postoperative tolerance curves in some instances is quite probable. However, the fact that the increased tolerance persists for a matter of weeks safely rules out dietary factors *per se* as being the primary causation variable.

The only as yet recognizable factor that was constant in all the instances where striking change in tolerance occurred was the direct accessibility of the lesion debris to the ventricular cerebrospinal fluid.[7] The close correlation between the duration of the disturbed tolerance and the time it takes for the organization of lesion debris may be significant.

The question is raised as to the possibility of the ultimate disturbance being a reversible impairment in liver function. This possibility is not too remote because drastic acute liver damage[8] and hepatic cirrhosis[9] have occasionally been encountered following experimental intracranial procedures.

Summary. A reversible decrease in glucose tolerance, variable in magnitude, has been encountered following major intracranial procedures in dogs.

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7 Keller, A. D., and D'Amour, M. C., *Arch. Path.*, 1936, **21**, 185.

8 Keller, A. D., *Arch. Path.*, 1936, **21**, 127.

9 Graef, I., Negrin, J., and Page, Irvine H., *Am. J. Path.*, 1944, **20**, 823.

15465

Adaptation of Colorado Tick Fever Virus to Mouse and Developing Chick Embryo

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Colorado tick fever is a human infection, probably of tick-borne viral etiology, which runs a mild course and is characterized by intermittent nonexanthematic fever. The disease has been recognized as a separate clinical entity by Becker under the name of Colorado tick fever[1] and by Toomey under the name of American Mountain Fever.[2] Topping, Cullyford and Davis[3] conducting a clinical and epidemiological survey of Colorado tick fever were unsuccessful in their attempts to establish the causative agent of the disease in laboratory animals or developing chick embryo. Recently, however, Florio and his colleagues[4] reported transmission of Colorado tick fever virus to the Syrian hamster and found that the virus passes gradocol collodion membrane filters with calculated pore diameters of 24 milli-microns.[5]

The present observation deals with adaptation of Colorado tick fever virus to the dba (dilute brown agouti) mouse, the white mouse and developing chick embryo.

Experimental. Infected hamster serum representing the 30th hamster passage of the virus was obtained through the courtesy of Dr. Lloyd Florio, of the School of Medicine and Hospitals, University of Colorado. The serum was diluted 5 times in saline and inoculated, respectively into: 5 hamsters by intraabdominal route, 6 dba mice by intranasal route and 12 dba mice by intracerebral route. The hamsters weighed 90-120 g and the mice 10-12 g. Two hamsters were found dead and 2 sick on the fourth day after inoculation. A low WBC count, characteristic for Colorado tick fever[4] was observed on blood smears obtained from the 2 sick animals.

None of the intranasally-inoculated mice showed any signs of illness. Nevertheless, one animal was sacrificed on the fifth day after inoculation and a bacteriologically sterile suspension of its lungs was instilled intranasally into a second group of 6 dba mice. Four of these mice were found dead on the 13th day after inoculation but all attempts to recover the causative agent from their lung tissue were futile and therefore no further attempts were made to carry the infection by the intranasal route.

Of the 12 mice inoculated intracerebrally with the infected hamster serum one was found dead on the fifth day after inoculation and one showed signs of illness on the seventh day after inoculation. The brains of these animals were removed aseptically and found to be sterile by suitable bacteriological tests. Ten per cent suspensions of the brain tissue of the 2 mice were inoculated intracerebrally into 6 albino Swiss and 6 dba mice respectively. Two of the Swiss mice and 3 of the dba mice showed signs of illness on the seventh and eighth days after inoculation. They were sacrificed and a 10% suspension of their brain tissue was again passed by the intracerebral route into other groups of Swiss and dba mice. The infection was thus established and carried continuously by means of intracerebral transfers. Starting with the sixth passage the infection produced 100% mortality in both the Swiss albino and dba mice.

Up to the present, the virus has been transferred through 23 consecutive brain to brain passages. The average survival time of mice inoculated with the 23rd mouse brain passage virus was 3.33 days as compared to 9.00 days for mice inoculated with the fourth passage.

1 Becker, F. E., *Colorado Med.*, 1930, **27**, 36, 87.

2 Toomey, N., *Ann. Int. Med.*, 1931-32, **5**, 585, 601, 912.

3 Topping, N. H., Cullyford, J. S., and Davis, G. E., *Pub. Health Rep.*, 1940, **55**, 2224.

4 Florio, L., Stewart, M. O., and Mugrage, E. R., *J. Exp. Med.*, 1944, **80**, 165.

5 Florio, L., Stewart, M. O., and Mugrage, E. R., *J. Exp. Med.*, 1946, **83**, 1.

TABLE I.
Identification of Colorado Tick Fever Mouse Brain and Chick Embryo Adapted Virus by Means of Intracerebral Neutralization Tests in Mice.

Virus				
Origin	Passage	Serum	LD_{50} titer	LD_{50} doses of virus neutralized
Mouse brain	16	Human C.t.f.* immune	$10^{-2.37}$	1349
		Human normal	$10^{-5.50}$	
" "	16	Hamster C.t.f. immune	$10^{-2.78}$	525
		Hamster normal	$10^{-5.50}$	
Chick embryo	10	Human C.t.f. immune	$10^{-1.25}$	224
		Human normal	$10^{-3.60}$	

* C.t.f.—Colorado tick fever.

The following symptoms of infection listed in order of their appearance, have been observed in mice: hyperexcitability, ruffled fur, sitting in a hunched position, labored breathing and general weakness. Paralysis was an exception rather than a rule and was never of the flaccid type. Death very often occurred within a few hours after the onset of symptoms.

Comparative titration of the 17th mouse brain passage virus in albino Swiss mice, 21-28 days of age, inoculated by intracerebral, intraabdominal and intranasal routes, respectively, resulted in the following LD_{50} titers: intracerebral route—$10^{-6.00}$, intraabdominal route—$10^{-1.85}$ (scattered deaths through several dilutions) and intranasal route—less than $10^{-1.00}$.

The virus circulates in the blood of an intracerebrally-injected mouse from the first day after inoculation until the death of the animal. Infectivity titration tests made with tissues removed at the peak of the disease have shown the brain and spinal cord to contain the greatest amount of virus, followed by heart muscle, spleen and lungs.

The virus readily passes through Berkefeld N and Seitz EK filters.

Identity of the virus. Identification of the virus adapted to mouse brain passage was based on: (a) neutralization by Colorado tick fever human and hamster antisera; (b) survival of reinoculated mice that resisted infection with the early mouse brain passages.

(a) Immune human serum was obtained from Dr. Florio (see above) who had Colorado tick fever about 3 years before the blood sample he sent us was obtained. Immune hamster serum was obtained from animals that survived inoculation with the original hamster passage virus. Human serum obtained from an individual who had never been in a Colorado tick fever endemic region, and a pool of normal hamster sera served as controls. Equal volumes of undiluted serum and serial 10-fold dilutions of infected mouse brain suspension (representing the 16th passage of the virus) were mixed, incubated for one hour in a water bath at 37°C and inoculated into mice by the intracerebral route. The results of the test shown in Table I indicate close relationship of the mouse brain adapted virus with the pathogenic agent which caused infection of the human individual and the hamsters.

(b) Nineteen mice that survived inoculation from the first and second mouse brain passages were reinoculated, 30 days later, with a 10% mouse brain suspension representing the 17th passage of the virus in mice. Twelve normal dba mice inoculated simultaneously as controls were found dead 5 to 7 days after injection. None of the 19 reinoculated mice showed any symptoms of disease.

Cross-neutralization tests. Preliminary studies were also conducted on neutralization tests between Colorado tick fever mouse brain virus and antisera against 2 other tick-borne diseases, *i.e.*, Russian spring-summer encephalitis and Louping-ill. Immune guinea pig sera, prepared against the latter 2 viruses, were obtained through the kindness of Dr. J. Casals of the Rockefeller Institute for Medical Research. The technic of the neutralization tests followed that described above. The results of the tests shown in Table II were completely negative in relation to Louping-ill immune serum. The Russian spring-summer encephalitis antiserum seemingly exerted a

TABLE II.
Results of Intracerebral Neutralization Test in Mice Using Colorado Tick Fever Mouse Brain Virus and Russian Spring-Summer Encephalitis, Louping-Ill and Colorado Tick Fever Immune Sera.

Serum	Animal source	LD_{50} titer of Colorado tick fever	LD_{50} doses of virus neutralized by sera
Russian Spring-Summer	guinea pig	$10^{-4.85}$	14
Louping-Ill	" "	$10^{-6.36}$	0
Colorado tick fever	hamster	$10^{-2.40}$	3981
Normal control	guinea pig	$10^{-6.0}$	

very slight or insignificant protective power.

Adaptation of the virus to developing chick embryo. Twenty 7-day-old chick embryos were inoculated by the yolk sac route with 0.25 ml of 1% mouse brain suspension representing the 12th mouse brain passage of the virus. Four living embryos were harvested on the fifth day after inoculation and a 10% suspension of the embryos was inoculated into another lot of 20 7-day-old embryos. Titration of the inoculum by the intracerebral injection of mice resulted in $10^{-3.5}$ LD_{50} titer. From the second passage on, a 10% suspension of living embryos, harvested on the fourth day after inoculation, has been routinely employed as passage inoculum. Up to now the virus has been carried through 12 passages. No deaths have been observed among the inoculated embryos. All attempts to demonstrate cultivable bacteria have been consistently negative. Chick embryo suspension, representing the tenth egg passage of the virus, was used as a source for neutralization tests against Colorado tick fever immune human serum of Dr. Florio (see above). The results of the tests, also summarized in Table I, indicate that the pathogen propagated in the developing chick embryo, seems to be closely related to the agent which caused infection of the human individual.

15466

A Toxic Principle from Progestational Endometrium and Placenta.

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Saline extracts of pregnant and pseudopregnant rabbit uteri have been shown to be highly toxic to rabbits when injected intravenously, while similar extracts of muscle, liver and nonpregnant uteri had no comparable effect.[1,2] Saline extracts of placenta have been similarly toxic.[3] The possibility that this induced intoxication might have some relation to toxemia of pregnancy has been expressed.[3-5] A further study of similar tissue extracts was undertaken in order to learn more about the nature of the toxic action and to determine more specifically the origin of the toxic substance. Extracts of different portions of the uterus in different functional states from several animal species, as well as extracts of liver, intestine and skeletal muscle, have been studied by a method for quantitative estimation of the toxic effect.

Method. The tissue was ground in a mortar with sand. The resultant paste was diluted

[1] Krichesky, B., and Pollock, W., *Am. J. Physiol.*, 1940, **130**, 319.

[2] Krichesky, B., and Mahler, J., *Endocrinology*, 1942, **30**, 616.

[3] Obata, I., *J. Immunology*, 1919, **4**, 111.

[4] Young, J., *J. Obs. and Gyn. Brit. Emp.*, 1914, **26**, 1.

[5] Bartholomew, R. A., and Kracke, R. R., *Am. J. Obs. and Gyn.*, 1932, **24**, 797.

with 0.9% NaCl solution and the sediment was removed by centrifugation. This sediment was again extracted with 0.9% NaCl solution and the separated supernatant fluid was added to the primary extract, which was then neutralized with N/10 NaOH until faintly alkaline. This solution, which was cell free, was adjusted so that 4 cc represented one g of source material; it was then stored at 0°C.

Young white mice were used as test animals. These were obtained from the colony maintained in the laboratory of the Department of Obstetrics and Gynecology at Stanford University. White mice from several other sources (including CFW), brown mice (agouti C_3H) and wild mice gave similar results. No sex difference has been observed.

Trial doses of the extract to be tested were given intravenously into the lateral tail veins of weighed mice until the minimum lethal dose for a 20 g mouse could be estimated by interpolation. The toxicity of the tissue extracts was expressed as the number of minimal lethal doses, or "units," per cubic centimeter. Ordinarily, about 8 mice were used for each assay. Repetition of assays gave results of similar magnitude, which usually checked within 10%. Estimations of toxicity of different dilutions of the same preparation (2.5-40 u/cc) demonstrated an inverse proportion between the dilution of the extract and the toxic effect.

Reaction of the test animals. After intravenous injection of toxic doses of active extracts the mice almost immediately became comatose and apnoeic. Some had generalized convulsions. Most either died promptly or apparently recovered within a few minutes. When an injection was fatal the heart continued to beat after respiratory movements had ceased. In a few animals, clotted blood was found in the mesenteric veins and in the vena cava soon after death. However, these could not be identified as ante-mortem clots, and no other anatomical findings explained the deaths. No hemorrhages or other lesions were seen in the brain. A few animals remained in collapse for as long as a few hours, after which there was death or gradual recovery. An occasional mouse, several hours after apparent recovery, developed violent convulsions and died.

In mice and rats, and apparently also in rabbits and dogs, an increase in tolerance could be built up by repeated injections of toxic extracts. After such preparation, animals survived the injection of 2 to 4 times the fatal dose. Within 2 days the acquired tolerance was completely lost in mice.

Light ether anesthesia during the injection did not modify the course of the reaction. Intramuscular, intraperitoneal and subcutaneous injections of 60 times the fatal intravenous dose failed to produce any response except for temporary reduction in the activity of the animals. An occasional mouse developed convulsions and died after repeated intramuscular injections.

Sources and properties of the toxic substance. Table I summarizes the results of assays of a variety of tissue types. All of the highly toxic extracts were derived from preparations which contained endometrium or placenta. Whether the relatively mild toxic effect of extracts of skeletal muscle on intravenous injection into mice represented the action of a substance identical to that found in the endometrium and placenta has not been determined.

The toxic substance was not extractable in ether, acetone or alcohol. It was lost upon heating extracts to 75°C for 10 minutes, addition of ethyl alcohol to a concentration of 25% or more, or upon acidifying the solution to pH 5. It did not dialyze through a cellophane membrane. Storage of the extracts even at low temperature resulted in a reduction of toxicity, which decreased as much as 50% in 48 hours.

Loss of toxicity of tissue extracts was considerably accelerated at room temperature by the addition of serum of mice or rats; a serum concentration of 5% caused a 50% decrease in toxicity within one hour. Serum of male as well as of female animals was effective. Toxic extracts of skeletal muscle, placenta and endometrium were all inactivated by serum. Heating the serum to 75°C for 10 minutes destroyed its inactivating property.

Several repeated injections of sublethal quantities of toxic extracts did not kill mice

TABLE I.

Approximate Toxicities of Extracts from Different Sources as Determined by the Mouse Assay. Those Designated as "0" contained less than 10 units per gram.

Source of extract	No. of extracts	Units per g of tissue
Endometrium		
Human, secretory phase	4	100
" 3 mo. pregnant	2	200
" menstrual discharge*	6	0
Cow, corpus luteum phase		
portion with papillae	1	50
" without "	1	50
Cow, normal diestrous	2	0
Sheep, " "	4	0
Rat, deciduoma†		
7 days	4	100
12 "	3	0
Endometrium with myometrium		
Rabbit, at estrus (Friedman test animal)	6	100-200
Rabbit, normal virgin uterus	2	0
Rat, pregnant	3	100-200
Deciduoma† uterus		
7 days	7	100-200
12 "	3	100-200
Deciduoma† uterus, with deciduoma core discarded		
7 days	4	100-200
12 "	3	100-200
Normal diestrous	1	0
Mouse, pregnant	3	100-200
" normal diestrous	4	20-100
Placenta, human, at term		
with endometrial layer	5	100-200
fetal portion only	5	100-200
Cow, near term‡	2	100-200
Sheep, " " ‡	4	100-200
Myometrium		
Human, secretory phase	3	0
" 3 mo. pregnant	2	0
Small intestine, rabbit	1	0
Rat	3	0
Mouse	1	0
Liver, rabbit	1	0
Rat	4	0
Mouse	2	0
Skeletal muscle, rabbit	1	0
Rat	3	20
Mouse	1	20

* Extracts of washed menstrual residue were no more active than extracts of the whole discharge.

† Necrosis began 7-8 days following induction and increased progressively.

‡ For anatomical reasons, endometrium remained mixed with cow and sheep placenta.

or rats, and it was found that the animals could tolerate as much as 4 times the lethal dose if the duration of the injection was prolonged to 90 seconds. This suggests that an animal is able to neutralize the toxic property of an injected extract very quickly, perhaps by means of an inactivating effect of plasma. Because of this rapid inactivation of the toxic property, it is necessary in assaying the material to make injections quickly and to avoid backflow of blood into the injecting syringe.

Discussion. Although the nature of the toxic principle found in extracts of endometrium or of placenta has not been determined, the properties are consistent with those of a protein. The toxic factor may be regarded as a specific substance or group of substances since it was not obtained by extraction of a number of other tissues and since it is rapidly inactivated by serum. It is probable that the substance described here is the same as that described as histamine-like by Krichesky and Pollock.[1] The properties of the material, particularly its failure to dialyze, support their conclusion that the substance is not histamine itself.

Since extracts prepared from myometrium were not toxic and since endometrium or placenta was present in all of the sources which produced strongly toxic extracts, it is probable that endometrium and placenta were the principal source of the toxic substance. Endometrium under progesterone influence, such as that in the secretory phase of the menstrual cycle, pseudo-pregnancy, or pregnancy, regularly produced a more toxic extract than endometrium in other physiological states.

It is doubtful whether the substance described here is the same as the menstrual toxin of Schick[6] or of Macht and Lubin.[7] Subcutaneous injection of extracts of menstrual discharge are reported by Smith and Smith[8] to result in local inflammation and in delayed death of injected animals. Extracts of menstrual discharge prepared and administered by the methods described here did not cause death in mice. However, it is possible that a toxic substance from the endometrium may have been inactivated in the discharge by the admixed blood.

[6] Schick, B., *Wien. Klin. Wchschr.*, 1920, **33**, 395.

[7] Macht, D. I., and Lubin, D. S., *J. Pharmacol. and Exp. Therap.*, 1924, **22**, 413.

[8] Smith, O. W., and Smith, G. S., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 285.

Summary and Conclusions. Toxic material, which causes death of mice on intravenous injection, can be extracted from progestational endometrium and from placenta of several mammalian species. Small amounts of material with a similar effect are present in extracts of skeletal muscle. Quantitative estimation of the toxicity of a variety of tissue extracts has been made. Addition of blood or serum rapidly diminishes this toxic property.

Special appreciation is due Miss Carol Spaulding for assistance in conducting this investigation.

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Liver Necrosis in Mice Following Injection of Toxic Extracts of Progestational Endometrium and of Placenta.

CHARLES L. SCHNEIDER. (Introduced by A. J. Cox, Jr.)

From the Department of Pathology, Stanford University School of Medicine, San Francisco, Calif.

During a study of the action of toxic extracts of endometrium and of placenta[1] a number of treated mice were found to have focal necrosis of the liver. Liver lesions have appeared in 174 of the animals; this represents an incidence of about 30% in mice which received sublethal amounts of toxic extract injected intravenously. The animals ranged from 1½ to 2 months of age. The lesions have been studied further by examining the livers of treated animals at different intervals following the injections.

Results. Within the first hour after injection, irregular areas of discoloration could be seen through the capsule of the liver. At first they were dark red, but after 24 hours they were usually paler than the adjacent liver tissue, and became sharply defined. Large lesions often presented a mottled appearance, with darker spots on a pale background (Fig. 1). The lesions were usually near the periphery of the lobes, and their size and number were variable. Usually the altered zones were small, and they occurred most frequently in the large left lobe, but all lobes were involved when the lesions were extensive. Occasionally, with massive lesions, there were fibrinous adhesions of the involved parts to adjacent structures. Small lesions were invariably covered by a smooth surface, although they were usually situated just beneath the liver capsule.

Microscopically there was dilatation of the hepatic sinusoids in the earliest lesions recognized, and erythrocytes were sometimes seen outside of vessels. Within 3 hours vacuolation of hepatic cells could be seen (Fig. 2) in the altered regions, and the nuclei were sometimes small and deeply stained. After 17 to 20 hours much of the cell structure had been destroyed (Fig. 3) in sharply demarcated zones, bordering which the adjacent hepatic cells were sometimes unusually deeply stained. At this stage there were fairly numerous polymorphonuclear leucocytes, particularly near the margin of the lesions.

The lesions had no constant position in the liver lobules. When the lesions were minimal, involving only small groups of cells, they were mid-zonal distribution. Sometimes entire lobules were necrotic, and the lesions commonly extended throughout several lobules or parts of several lobules without apparent predilection for any part.

In several animals there were tiny pedunculated lobes of liver tissue protruding caudally near the right kidney. These all showed necrosis in treated animals, even in the absence of lesions elsewhere. Several recent thrombi in vessels within the narrow stalk which attached these structures to the

1 Schneider, C. L., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 322.

Fig. 1.
Abdominal organs of a mouse killed 17 hours after intravenous injection of an extract of rabbit uterus at estrus. The mottled portions of the liver indicate the necrotic zones.

Fig. 2.
Histological section of a mouse liver removed 1½ hours after injection of a toxic extract of rabbit uterus. In the upper half of the photograph are many abnormal vacuolated cells.

liver suggested a possible mechanism for the formation of these lesions. Thrombi were also found in small veins adjacent to other areas of liver necrosis, although they were usually not demonstrable in association with small lesions.

The time required for absorption of the necrotic liver was dependent upon the size of the lesion. Necrotic cells sometimes persisted for 4 days or longer. Mitotic figures could usually be seen in the surrounding hepatic tissue after 2-4 days. There was relatively little growth of fibrous tissue, which was pronounced only at the periphery of the large lesions.

The sex of the mice, a preliminary period of starvation or deprivation of water, a meat diet for 2 days, and variations in the character of the anesthetic at the time of injection, had no recognizable influence upon the development of the liver lesions.

Injections of only slightly less than the fatal quantity of toxic extract were most frequently followed by liver damage, and under these conditions the lesions were often large. However, liver necrosis did not occur invariably in such animals, and lesions developed in some mice after injection of much less than a lethal quantity of an active extract. The liver necrosis, like the acute death of mice, occurred regularly only after intravenous injection of the toxin.

When toxic extracts were mixed with serum *in vitro* there was rapid diminution of the toxic property as measured by the increased amount of the extract necessary to produce death of mice.[1] Injection of sublethal quantities of this partially detoxified extract

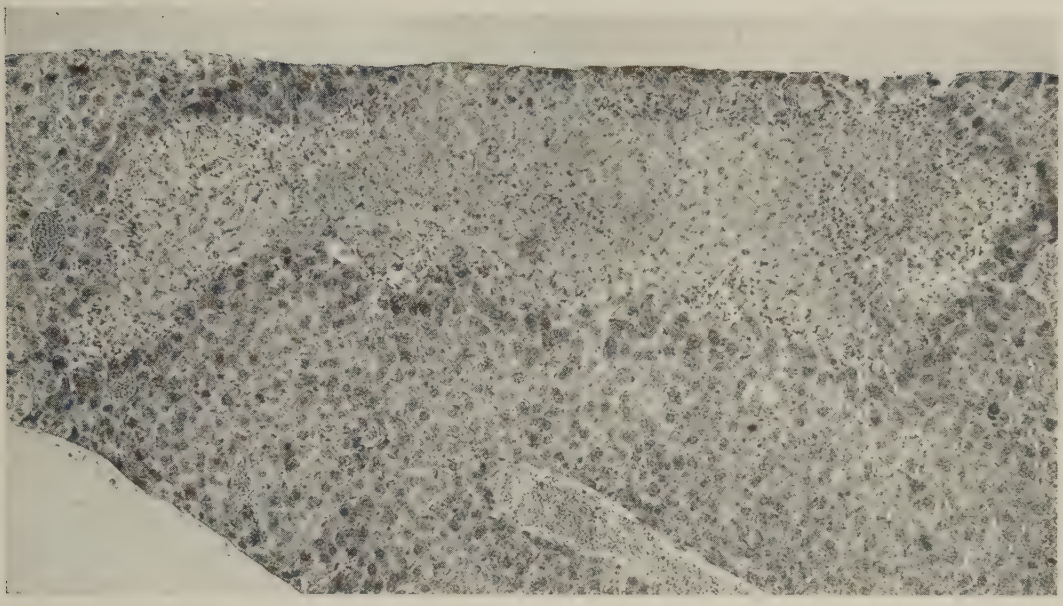

Fig. 3.
Histological section of a mouse liver removed 20 hours after injection of a toxic uterine extract, showing a zone of necrosis adjacent to the liver surface.

did not produce liver lesions more frequently than did much smaller amounts of untreated extract. Similarly, slow injection of several times the quantity which was lethal on rapid injection did not increase the incidence of lesions. These observations suggest that the production of liver lesions is related to the ability of the extract to kill animals, and that serum or plasma influences both properties.

In 203 control mice, most of which had been given quickly fatal injections of toxic uterine extracts, grossly visible liver lesions similar to those in the experimental animals were found in 3 instances. None of these had received injected extract long enough prior to death to account for the lesions. An occasional animal injected with extracts of other types of tissue also showed liver lesions, although the only tissue to elicit the change with a frequency distinctly greater than that of the control group was skeletal muscle. Of 69 mice injected with muscle extracts, 14 developed liver lesions.

Most of the mice with liver lesions showed no other gross tissue changes. Likewise, histological sections of kidney, spleen, heart, lung, thyroid, adrenals and brain from several of the animals disclosed no abnormalities. In a few instances there was hemorrhage in one or more tissues, including the intestine, mesenteric lymph nodes, lungs and eye. No underlying lesion was detected. Several thrombi were found on the atrial endocardium.

Five rabbits and 4 dogs which survived intravenous injections of toxic extracts showed no liver necrosis. Of 51 rats surviving intravenous injections only 2 had liver necrosis similar to that of the mice.

Discussion. Krichesky and co-workers[2,3] did not mention liver necrosis in their rabbits injected with toxic extracts of pregnant or pseudopregnant uteri, but focal liver necrosis has been reported by various investigators using placental extract in an effort to produce experimental toxemia of pregnancy. Young[4] with suspensions of dried, autolysed, powdered placenta injected hypodermically, obtained liver necrosis in 40% of 15 guinea pigs. Others were reported to have "degenerative lesions."

2 Krichesky, B., and Pollock, W., *Am. J. Physiol.*, 1940, **130**, 319.

3 Krichesky, B., and Mahler, J., *Endocrinology*, 1942, **30**, 616.

4 Young, J., *J. Obs. and Gyn. Brit. Emp.*, 1914, **26**, 1.

Obata[5] observed lesions in 3 of 12 rabbits injected intravenously with saline extracts of fresh placenta. He mentioned hemorrhagic liver lesions in his injected mice. Oden[6] upon repeating the methods of Young with 3 guinea pigs and 7 rabbits, declared that the liver lesions were diffuse, with "no lesions in any manner resembling those of eclampsia." No mention was made of liver lesions in his mice that received injections of extract. Bartholomew and Kracke[7] reported liver and kidney lesions in rabbits and guinea pigs.

From these reported observations and from the circumstances leading to the development of the liver lesions here reported, it is apparent that there is a relationship between the development of focal liver necrosis and the injection of toxic extracts of endometrium and of placenta. However, the appearance of a few similar foci of necrosis in animals which received injections of extracts of a variety of tissues, or occasionally too soon after injection to be a result of the treatment, suggests that the experimental lesions may not result directly from the action of the extract, but may follow activation of some latent process. Since most of the changes in the liver developed after the animals that received injections of extract had suffered a temporary collapse or shock-like state, this non-specific influence may have been a factor in the pathogenesis of the lesions. The possibility that the lesions are identical with the spontaneous focal liver necrosis in mice reported by Olitsky and Casals[8] has been considered, but the lesions described here were grossly visible and involved larger zones than did the spontaneous lesions. No inclusion bodies have been found in any of the sections of liver from animals used in this study.

Summary. Focal liver necrosis occurred in 30% of 500 mice which survived the intravenous injection of single sublethal quantities of toxic extracts of endometrium or placenta. Occasional similar lesions were seen in mice which were injected with other tissue extracts. Although related to the effect of the injected toxic material from endometrium or placenta, the lesions may be indirect or nonspecific effects of the injections.

Special appreciation is due Miss Carol Spaulding for assistance in conducting this investigation.

[5] Obata, I., *J. Immunology*, 1919, **4**, 111.

[6] Oden, C. L. A., *J. Mich. Med. Soc.*, 1925, **24**, 110.

[7] Bartholomew, R. A., and Kracke, R. R., *Am. J. Obs. and Gyn.*, 1932, **24**, 797.

[8] Olitsky, P. K., and Casals, J., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 48.

15468 P

Influence of Dietary Iodine on Susceptibility of Rats to Alpha Naphthylthiourea Poisoning.*

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Richter[1] proposed the use of α-naphthylthiourea (ANTU) as a rodenticide for the control of Norway rats because of its high toxicity to carnivorous species and its relatively low toxicity to omnivorous and herbivorous species.

While it has not yet been reported that any

* This work was carried out under contract with the Medical Division of the Chemical Warfare Service.

† The experimental data in this paper are taken from a thesis submitted *in absentia* in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate School of the University of Illinois.

[1] Richter, C. P., *J. A. M. A.*, 1945, **129**, 927.

dietary factor will influence an animal's resistance to ANTU, Griesbach *et al.*[2] have shown that iodide, a normal dietary component, when injected in doses of 1.30 mg per rat every 4 days protected the animals against poisoning by thiourea, the parent compound from which ANTU is derived. DuBois and Erway[3] have demonstrated that ANTU and related compounds inhibit tyrosinase, and that this inhibition can be prevented by iodine.

Therefore, a study of the effect of iodine fed in the diet on the resistance of rats to ANTU poisoning was undertaken.

Methods and Materials. Three- to 4-month-old albino rats of the Sprague-Dawley strain averaging 200 g in weight were used throughout these experiments. Iodine was given to the animals either dissolved in the drinking water or mixed into the diet. Both food and water, containing iodine, were provided *ad libitum* but the actual iodine intake was recorded for each rat individually. The animals had eaten Purina Laboratory Chow for at least 3 weeks before receiving the experimental diets.

The stock diet used in these experiments contained: casein 18%, cerelose 15%, dextrin 50%, cellu flour 2%, salt[4] 3%, lard 4%, corn oil 4%, and cod liver oil 4%. A vitamin supplement containing thiamine hydrochloride 250 γ, pyridoxine hydrochloride 250 γ, nicotinic acid 250 γ, riboflavin 500 γ, calcium pantothenate 2.5 mg, choline chloride 125 mg, α-tocopherol 10 mg, and Wilson's 1-20 liver concentrate 625 mg was added per 100 g of diet.

In experiments in which iodine was supplied in the drinking water it was given in dose equivalent to 2.52 mg I_2 per cc in the form of both Lugol's solution and potassium iodide.

After iodine feeding for various periods, the animals were injected intraperitoneally with ANTU dissolved in anhydrous propylene glycol.

Results. The effect upon the resistance of rats to ANTU of feeding iodine *ad libitum* at different concentrations in the drinking water and in the diet is shown in Table I.

TABLE I.
Relationship of Iodine Ingested in the Diet to Resistance of Rats to ANTU.

Avg I_2 intake mg/kg	ANTU mg/kg	Mortality	LD_{50} mg/kg
	4	0/5	
Purina Chow control	6	3/5	ca 6.5
	7	4/7	
	8	2/2	
	3	0/4	
	5	4/11	
0.2	7	2/7	" 7.0
	8	3/4	
	9	4/4	
	12	4/4	
	20	1/3	
	23	1/6	
20.8 (7.6-29.0)	26	1/4	" 29
	29	5/9	
	32	6/8	
	40	3/3	
	20	0/3	
	23	0/2	
	26	0/3	
	28	0/5	
44.8 (31-81.6)	32	1/7	" 46
	35	1/5	
	40	2/6	
	50	3/4	
	60	5/5	

After a 1-2-day feeding period a difference was noted in the protection provided by diluted Lugol's solution, potassium iodide in the water, or potassium iodide given in the diet. This difference was directly related to the quantity of iodine the animals had ingested. Those animals eating the greatest amount of iodine during the period were the most resistant to the rodenticide. It was noted that the intake of Lugol's solution at the concentrations used was 9 cc per day compared with an average intake of 16 cc per day by controls. Animals which drank water containing potassium iodide consumed an average of 14 cc per day. Animals receiving potassium iodide in the diet ate an average of 12 g of the food per day as compared with

[2] Griesbach, W. E., Kennedy, T. H., and Purves, H. D., *Nature*, 1944, **154**, 610.

[3] DuBois, K. P., and Erway, W. F., personal communication.

[4] Hubbel, R. B., Mendel, L. B., and Wakeman, A. J., *Nutrition*, 1937, **14**, 273.

13 g during the same period for the controls.

It will be noted that the dose of ANTU required to kill 50% of the animals (LD_{50}) was about 7.0 mg per kg for rats receiving the synthetic stock diet for 30 days. This value is not significantly different from an LD_{50} of 6.5 mg per kg for animals eating Purina Laboratory Chow for at least 3 weeks.

The rats receiving increased amounts of iodine in food and water were divided into 2 groups according to the quantity of iodine consumed. The average iodine intakes for the 2 groups expressed as mg iodine were 20.8 and 44.8 mg per kg. The LD_{50} for the group of animals which had ingested an average of 20.8 mg per kg of iodine during a 1-2-day feeding period was 29 mg per kg of ANTU, while the LD_{50} for animals having an average intake of 44.8 mg per kg of iodine over the feeding period was 46 mg per kg of ANTU. The animals were, therefore, able to tolerate some 4 to 7 times the amount of ANTU required to kill the controls. It is apparent that within these limits of iodine intake, the amount of iodine ingested over a 1-2-day feeding period increases the resistance of rats to the rodenticide.

Summary. This study has shown that iodine given either as Lugol's solution or as potassium iodide protected rats against ANTU poisoning. The ability of the animals to tolerate ANTU was proportional to the quantity of iodine which had been ingested over a 1-2-day period.

ANNUAL REPORT OF THE SECRETARY
April 1, 1945 to March 31, 1946

The national officers and committees of the Society held their first meetings since the war in Atlantic City March 11-12, 1946. These meetings were extremely well attended and the spirit of cooperation was splendid.

The following concise statements may give our members some idea of some important decisions reached by the Council.

Finances. There was for the fiscal year 1945-46 a surplus of $3400. This surplus occurred in spite of increasing costs of publication, and reduction in cost to authors. It was due partly to the large number of subscriptions by the U. S. Armed Forces for their medical centers. Because of this surplus the Council voted (1) to consummate its policy of progressive reduction in costs to authors, by making no charge for publication (except reprints, sold at cost); (2) to give authors 25 free reprints; (3) to send the Proceedings without cost for one more year to members and subscribers in Allied War zones; (4) to send the Proceedings to other institutions in stricken areas, also without cost.

Since the Atlantic City meetings our printer has informed us that in view of increased labor costs he would have to charge about $2400 a year more. We are informed that a similar increase in costs of printing is nation-wide. The Council reluctantly voted therefore, to modify its decisions, viz., that *if and when a deficit is imminent:*

1. Authors shall be charged 10% of cost for text and 25% of cost for illustrations.

2. *No* free reprints to be given to authors.

3. The Proceedings shall, however, be sent to members and subscribers in Allied war zones, upon their request, for another year without charge.

4. The Proceedings shall also be sent without charge for one year to libraries chosen by a committee appointed by the President (Doctors Engle, Fulton, Goldforb and Lambert). These libraries are in the bombed or stricken war zones.

Appeal for Back Numbers. After the annual meetings in March, information was received that (1) many Proceedings to Allied areas were lost by sinkings during the war, and requests were received for their replacement. (2) Due to errors and efforts to conserve paper, our stocks in certain numbers were extremely low or nil.

An appeal was therefore sent to members and subscribers to give or to sell us at cost those issues where our stocks were inadequate to meet our commitment. The response was most heartening. Nearly all Proceedings were sent as gifts. We are paying transportation costs, and notifying senders of the receipt of their packages. Members are requested to do all they can to send, or to get others to send, by express collect to the Griffiths Press, 100 Liberty Street, Utica, N. Y., the numbers given in the following list.

List I includes numbers where our stocks are nil.

List II includes numbers where our stocks are very low.

Year	Vol.	Month	*List I* No.	*List II* No.	Month
1940	43	Mar.	3		
	44	May	1		
	45	Oct.	1		
				2	Nov.
		Dec.	3		
1941	46	Jan.	1		
		Feb.	2		
		Mar.	3		
		Apr.	4		
	47	May	1		
				2	June
	48	Oct.	1		
				3	Dec.
1942	49			1	Jan.
				2	Feb.
				3	Mar.
				4	Apr.
	50			1	May
	51	Oct.	1		
		Nov.	2		
		Dec.	3		
1943	52			1	Jan.
		Feb.	2		
		Mar.	3		
		Apr.	4		
	53	May	1		
		June	2		

We are mailing these Proceedings as promptly as possible.

Finance Committee. The Society is much indebted to the members of the Finance Committee and to their unofficial expert advisors for the extremely able and ever watchful care of the Society's investments. Special thanks are due the Treasurer's advisor on investments, Mr. H. G. Friedman. In conformity with the Council's policy of rotation, Dr. C. D. Leake,

who served so many years and so splendidly was succeeded by Dr. Philip Shaffer.

Membership. Upon recommendation of the National Membership Committee the Council approved modifications of the by-laws. These changes it is believed will minimize delays and difficulties experienced in the past. The proposed by-laws were approved almost unanimously by the large number of members voting. Article 8, Section V, as adopted reads as follows. Deletions are indicated by ( ), additions in *italics.*

Art. 8, Section V. Candidates for membership in the Society shall be proposed by three members and such proposal shall have endorsed thereon the approval of the officers or membership committee of the Section. *Such proposal shall be made to the National Membership Committee of the Society. If 6 out of 9 members of this Committee approve an application, the applicant is elected. If 5 members of the Committee approve, the application shall be sent to the Section Secretary for further information and then referred back to the National Membership Committee for reconsideration. If the vote of this Committee is still 5/9, the application will be referred to the Council, for final decision. The Council may, by a 2/3 vote elect such applicant to membership.* Nominations must be received by the Secretary-Treasurer (by December 15) for transmission to the National Membership Committee. The Membership Committee shall meet annually (to consider such applications). Their report shall be forwarded to the Council (for final action. A two-thirds vote of the Council is necessary for election.)

With the adoption of these changes it is expected that (1) applications will be acted on far more promptly; (2) few applications will need to be referred back to the Committee for reconsideration; (3) and still fewer referred to the Council. To expedite action further the Membership Committee requested Dr. Hodges to formulate a new application form that would request authors and their sponsors to give precise and brief answers to questions, establishing eligibility of applicant to membership. Applicants may make nine copies to be sent simultaneously to all members of the Committee.

Emeritus Members. The following were elected by the Council to emeritus membership: Doctors E. C. Schneider, J. W. Jobling, Florence Sabin, Mary B. Stark, C-E. A. Winslow, W. H. Woglom.

Deaths. The Council records with sorrow the death of the following members: Doctors Leon Asher, M. Bergmann, H. O. Calvery, W. B. Cannon, C. B. Davenport, G. S. deRenyi, A. G. Eaton, S. Flexner, E. Harde, G. A. Harrop, T. H. Morgan, G. W. Raiziss, G. B. Ray, C. L. A. Schmidt, and A. T. Shohl.

Magnuson-Kilgore Compromise Bill S-1850. A copy of this bill together with a statement of the "Committee supporting the Bush Report" and a further "statement concerning Bill S-1850 were sent to the Council. Each Section was requested to send to its Federal senators a resolution supporting this bill. The Council voted, almost unanimously, to do the same on behalf of the entire Society.

Anti-Vivisection. Upon recommendation of the Southwestern Section the Council approved the following resolution. A copy will be available to any member of the society.

WHEREAS, experimentation with animals under satisfactorily controlled conditions is necessary for the advancement of knowledge regarding living things, and

WHEREAS, such knowledge contributes enormously to the health and happiness of people everywhere, and

WHEREAS, such experimentation is continually subject to the threat of legislative interference,

NOW BE IT RESOLVED by the Society for Experimental Biology and Medicine that respectful request be made to proper state and national legislative bodies, through the appropriate committees thereof, to assure the freedom of responsible scientific research in biology and medicine involving animal experiments, providing always that the animals in question are properly cared for and required to suffer no unnecessary pain.

The Council furthermore voted to send $500 to the National Society for Medical Research.

Secretary requested to tour South America. The Council unanimously voted that "the Secretary should be requested to make a study in Mexico and South American countries, to discuss eligibility to membership, standards of publication, exchanges, and possible free subscriptions to our Proceedings." It was voted to allow him $2500 to defray expenses of travel.

New Editors. In conformity with the Society's rotation policy, the following Editors' terms

of office expired, at the end of five years' service: Doctors Bieter, Engle, Francis, Gerard, Mattill, Rich, Sendroy and Shannon. The Society is greatly indebted to each of them for their conscientious, unbiased, able and prompt service. Members can hardly be aware of the huge task which they have so ably performed.

The Council elected the following to succeed them: Doctors R. E. Shope, C. P. Berg, W. M. Allen, A. D. Welch, N. Kleitman and B. Lucké for five years each, and Doctors C. A. Dragstedt and J. Sendroy for two years each.

Dr. Emil Bauman has continued to index so ably and so promptly each successive volume of the Proceedings. We are all greatly in his debt.

SECTIONAL MEETINGS AND MEMBERSHIP

Cleveland, Ohio

Chairman: W. Heymann Secretary: M. Miller Members: 53
Meetings: Western Reserve University, October 12, 1945
February 8, 1946
March 8, 1946
April 12, 1946

District of Columbia

Chairman: Henry Stevens Secretary: R. A. Cutting Members: 92
Meetings: George Washington University, December 6, 1945
February 7, 1946

Illinois

Chairman: C. I. Reed Secretary: W. S. Hoffman Members: 199
Meetings: University of Chicago, November 28, 1945
February 26, 1946
May 21, 1946

Iowa

Chairman: E. D. Warner Secretary: E. L. DeGowin Members: 47
Meetings: State University of Iowa, October 23, 1945
February 26, 1946

Minnesota

Chairman: R. G. Green Secretary: B. Campbell Members: 64
Meetings: University of Minnesota, December 19, 1945
February 26, 1946
April 29, 1946
May 28, 1946

Missouri

Chairman: L. R. Jones Secretary: C. F. Cori Members: 62
Meetings: Washington University, May 8, 1946

New York

Chairman: W. S. Root Secretary: W. M. Copenhaver Members: 527
Meetings: New York Academy of Medicine, December 19, 1945
February 20, 1946
May 22, 1946

Pacific Coast

Chairman: M. Kleiber Secretary: H. S. Olcott Members: 134
Meetings: University of California, September 1, 1945
Stanford University, October 24, 1945
University of California, December 12, 1945
Western Regional Research Laboratory, February 13, 1946
Stanford University, April 27, 1946

Peiping, China

No report on Peiping Section.

Rocky Mountain

Chairman: L. P. Gebhardt Secretary: R. M. Hill Members: 33
Meetings: University of Colorado, December 7, 1945
Colorado A. and M. College, March 16, 1946

Southern

Chairman: R. G. Smith Secretary: C. Reynolds Members: 55
Meetings: Tulane University, November 9, 1945
Louisiana State University, March 1, 1946
Tulane University, May 24, 1946

Southern California

Chairman: B. Krichesky Secretary: J. W. Mehl Members: 57
Meetings: California Institute of Technology, November 9, 1945
University of Southern California, February 20, 1946
University of California, Los Angeles, May 23, 1946

Southwestern

Chairman: D. Slaughter Secretary: R. H. Rigdon Members: 62
Meetings: Southwestern Medical College, October 10, 1945
University of Oklahoma, December 27, 1945
University of Arkansas, May 25-26, 1946

Western New York

Chairman: J. B. Sumner Secretary: A. H. Hegnauer Members: 73
Meetings: Strong Memorial Hospital, Rochester, November 17, 1945
University of Rochester, February 16, 1946
Cornell University, May 11, 1946

Wisconsin

Chairman: H. P. Rusch Secretary: W. H. McShan Members: 50
Meetings: University of Wisconsin, February 27, 1946

MEMBERSHIP

Members, March 31, 1945	1872	
Elected during year	160	
		2032
Resignations	10	
Arrears	4	
Deaths	12	
		26
Total Membership, March 31, 1946		2006

Membership:	1936	1946
	1325	2006

Subscriptions, March 31, 1946 1306

ANNUAL REPORT OF THE TREASURER

April 1, 1945-April 1, 1946

President Hastings appointed Doctors Hans T. Clarke, E. F. DuBois, and C. McEwen as Auditing Committee to examine the accounts of the Treasurer. This Committee requested Mr. Alexander Dolowitz, C.P.A., to go over the books of the Society and report to the Committee. The following is the statement of the Committee:

The members of the Auditing Committee appointed by Dr. Hastings have examined the Audit Report of the books of the Society for the period April 1, 1945 to March 31, 1946 and have expressed to me their unanimous approval of the report.

(Signed) HANS T. CLARKE

Statement of Income and Disbursements

April 1, 1945 to March 31, 1946

INCOME

Dues		$ 7,776.24	
Subscriptions	$11,876.75		
Reprints	2,412.95		
Excess Space	202.78		
Cuts	174.91		
Changes	8.77		
Back Numbers	1,322.18		
		15,998.34	
Interest—Special accounts		2.05	
Miscellaneous		32.95	
Total Income			$23,809.58

DISBURSEMENTS

Office supplies, telephone and postage		$ 746.73	
Cost of printing	$10,533.47		
Cost of reprints	2,358.98		
Cuts	1,046.32		
Storage and insurance	73.07		
Refunds	1,141.14		
Back numbers (Purchases)	7.50		
		15,160.48	
Salaries		3,629.90	
Miscellaneous		930.65	
Total disbursements			20,467.76
Excess of income over disbursements			$ 3,341.82

Society for Experimental Biology and Medicine

STATEMENT OF ASSETS AND LIABILITIES

AS AT MARCH 31, 1946

Assets

Cash in banks	$ 9,630.20	
Investments—Surplus Fund	32,188.61	
Accounts receivable	1,312.91	
Total Assets		$43,131.72

Liabilities

Accounts payable	$ 15.49	
Withholding taxes	123.50	
New York Section	215.22	
Total liabilities		354.21
Net worth		$42,777.51

Endowment Fund

N. Y. Title and Mortgage Certificate No. F1691	$ 4,800.00	
Title Guarantee & Trust Co	114.01	
Lawyers Mortgage Company	98.28	
Bowery Savings Bank	2,817.33	
R. R. Federal Savings and Loan	78.40	
U. S. Government Bonds	12,150.00	
Industrial Bonds	1,848.62	
Total		$21,906.64

MEMBERS' LIST

Abels, J. C. — Memorial Hosp., N. Y.
Abramson, D. I. — Galesburg, Ill.
Abramson, H. A. — Coll. Phys. and Surg., N. Y.
Abreu, B. E. — Univ. of Cal. Med.
Abt, Arthur F. — Northwestern Univ.
Acevedo, D. — Univ. de San Marcos, Peru
Adams, A. Elizabeth — Mount Holyoke Coll.
Adams, J. M. — Univ. of Minn.
Addis, Thomas — Stanford Univ. Med.
Adlersberg, D. — Beth Israel Hosp., N. Y.
Adolph, E. F. — Univ. of Rochester Med.
Adolph, W. H. — Cornell Univ.
Adriani, J. — Louisiana State Univ.
Albanese, A. A. — N. Y. U. Med.
Albaum, H. G. — Brooklyn Coll.
Alexander, Harry L. — Washington Univ.
Alexander, Hattie E. — Babies Hosp., N. Y.
Algire, G. H. — National Cancer Inst.
Allen, Bennet M. — Univ. of Calif., L. A.
Allen, Frank W. — Univ. of Calif.
Allen, J. G. — Univ. of Chicago
Allen, Willard M. — Washington Univ. Med.
Allen, William F. — Univ. of Oregon
Alles, G. A. — Univ. of Calif.
Allison, J. B. — Rutgers Univ.
Almquist, H. J. — Emeryville, Calif.
Alt, Howard L. — Northwestern Univ. Med.
Althausen, T. L. — Univ. of Calif. Med.
Altschule, A. M. — South. Reg. Res. Lab., New Orleans
Altschule, M. D. — Beth Israel Hosp., Boston
Altshuler, S. S. — El Paso, Texas
Alvarez, Walter C. — Mayo Clinic
Alving, A. S. — Univ. of Chicago
Amberg, Samuel — Mayo Clinic
Amberson, W. R. — Univ. of Maryland Med.
Ambrose, A. M. — West. Reg. Res. Lab., Albany, Calif.
Amoss, Harold L. — Rockefeller Inst.
Andersch, M. A. — Univ. of Maryland Med.
Anderson, Dorothy H. — Coll. Phys. and Surg., N. Y.
Anderson, H. H. — Univ. of Calif. Med.
Anderson, John A. — Univ. of Utah Med.
Anderson, John E. — Univ. of Minn.
Anderson, Richmond K. — Health Dept., Georgia
Anderson, Rudolph J. — Yale Univ.
Anderson, William E. — Rockville, Conn.
Andervont, H. B. — National Cancer Inst.
Andrew, Warren — Southwestern Med.
Andrus, E. C. — Johns Hopkins Univ.
Andrus, W. deW. — Cornell Univ. Med. Coll.
Angerer, C. A. — Ohio State Univ.
Angevine, D. M. — Wilmington, Del.
Anigstein, Ludwik — Univ. of Texas Med.
Ansbacher, Stefan — New York City
Antopol, William — Beth Israel Hosp., Newark, N. J.
Apperly, Frank L. — Med. Coll. of Va.
Aring, C. D. — Univ. of Calif. Med.
Armstrong, Charles — Nat. Inst. of Health, Washington
Armstrong, W. D. — Univ. of Minn.
Arnold, Lloyd — Univ. of Ill.
Arnow, L. E. — Glenolden, Pa.
Aron, H. C. S. — Northwestern Univ. Med.
Aronson, J. D. — Henry Phipps Inst.
Artom, Camillo — Bowman Grey Med.
Asdell, S. A. — Cornell Univ.
Ashby, W. M. — Washington, D. C.
Ashman, Richard — Louisiana State Univ.
Ashworth, C. T. — Southwestern Med.
Asmundson, V. S. — Univ. of Calif.
Atchley, D. W. — Presbyterian Hosp., N.Y.C.
Aub, Joseph C. — Mass. Gen. Hosp., Boston
Auer, John — St. Louis Univ.
Austin, J. Harold — Univ. of Pa.
Avery, B. F. — Boston Univ.
Avery, O. T. — Rockefeller Inst., N. Y. C.
Avery, Roy C. — Vanderbilt Univ.
Aycock, W. L. — Harvard Med.
Ayo, C. — Montefiore Hosp., N. Y.

Babkin, Boris P. — McGill Univ.
Bachem, Albert — Univ. of Ill. Med. Coll.
Baehr, George — Mt. Sinai Hosp., N.Y.C.
Baernstein, H. D. — National Inst. of Health
Bagg, Halsey J. — Yorktown Heights, N. Y.
Bahrs, Alice M. — Portland, Ore.
Bailey, Cameron V. — N. Y. Post-Graduate Med.
Bailey, Percival — Univ. of Illinois Med.
Baitsell, George A. — Yale Univ.
Bakwin, Harry — N. Y. Univ. Med. Coll.
Baldwin, Francis M. — Univ. of S. Calif.
Baldwin, I. L. — Univ. of Wisconsin
Ball, G. H. — Univ. of Calif., L. A.
Ball, H. A. — San Diego, Calif.
Balls, A. K. — West. Reg. Res. Lab., Albany, Calif.
Banerjee, S. — Calcutta, India
Barach, Alvan L. — Coll. Phys. and Surg., N. Y.
Barber, W. Howard — New York Univ. Med.
Bard, Philip — Johns Hopkins Univ.
Barer, Adelaide P. — State Univ. of Iowa
Barker, S. B. — Iowa City, Ia.
Barlow, O. W. — Rensselaer, N. Y.
Barnes, R. H. — Glenolden, Pa.
Barnes, T. C. — Hahnemann Med.

Barnett, George D. Stanford Univ.
Barnum, C. P., Jr. Univ. of Minn.
Barr, David P. Cornell Med. Coll.
Barron, E. S. G. Univ. of Chicago
Barth, L. G. Columbia Univ.
Bartley, S. H. Dartmouth Coll.
Bass, Allan D. Syracuse Univ. Med.
Bass, Charles Tulane Univ.
Bassett, D. L. Stanford Univ.
Bast, T. H. Univ. of Wisconsin
Bateman, J. B. Mayo Clinic
Bates, R. W. New Brunswick, N. J.
Batterman, R. C. New York Univ. Med.
Bauer, J. H. Rockefeller Fdtn., Paris
Bauman, Louis Presbyterian Hosp., N.Y.C.
Baumann, Carl A. Univ. of Wisconsin
Baumann, E. J. Montefiore Hosp., N.Y.C.
Baumberger, J. Percy Stanford Univ.
Bayley, R. H. Univ. Okla. Med.
Bayne-Jones, S. Yale Univ.
Bazett, H. C. Univ. of Pa.
Beams, H. W. State Univ. of Iowa
Bean, John W. Univ. of Mich.
Beard, J. W. Duke Univ.
Beck, Claude S. Western Reserve Univ.
Becker, E. R. Iowa State Coll.
Becker, R. F. Washington Univ. Med.
Beckman, Harry Marquette Univ. Med.
Beckwith, T. D. Univ. of Calif., L. A.
Beeson, P. B. Emory Univ.
Behre, Jeannette A. New York City
Belding, David L. Boston Univ.
Bender, M. B. Mt. Sinai Hosp., N. Y.
Bengston, Ida A. National Inst. of Health, Washington
Bennett, G. A. Univ. Illinois Med.
Bennett, L. L. Univ. of Calif.
Berg, B. N. Columbia Univ.
Berg, C. P. State Univ. of Iowa
Berg, William N. New York City
Bergeim, Olaf Univ. of Ill.
Bernhard, Adolph Lenox Hill Hosp., N.Y.C.
Bernhart, F. W. Chagrin Falls, O.
Bernheimer, A. W. N. Y. U. Med.
Bernthal, T. G. Vanderbilt Univ.
Berry, George P. Univ. of Rochester Med.
Best, C. H. Univ. of Toronto
Beutner, R. Hahnemann Med. Coll., Philadelphia
Beyer, K. H., Jr. Glenolden, Pa.
Bierman, W. Mt. Sinai Hosp., N. Y.
Bieter, Raymond N. Univ. of Minn.
Bills, C. E. Mead, Johnson and Co., Evansville, Ind.
Bing, Franklin C. Am. Med. Assn., Chicago
Bing, R. J. Johns Hopkins Univ.
Birkhaug, Konrad E. Geofysisk Inst., Bergen, Norway
Birnbaum, G. L. New York Med. Coll.
Bishop, George H. Washington Univ. Med.
Biskind, G. R. Mt. Zion Hosp., San Francisco
Bittner, J. J. Univ. of Minn. Med.
Blair, John E. Hosp. for Joint Diseases, N.Y.
Blake, F. G. Yale Univ.
Blalock, Alfred Johns Hopkins Univ. Med.
Blatherwick, Norman R. Metropolitan Life Ins. Co., N. Y. City
Blinks, L. R. Stanford Univ.
Bloch, Robert G. Univ. of Chicago
Block, Richard J. N. Y. Med. Coll.
Block, Walter D. Univ. of Michigan
Bloom, William Univ. of Chicago
Bloomfield, A. L. Stanford Univ. Med.
Bloor, W. R. Univ. of Rochester
Blount, R. F. Univ. of Texas Med.
Blum, Harold F. National Cancer Inst.
Blumberg, Harold Rensselaer, N. Y.
Blumgart, H. L. Beth Israel Hosp., Boston
Bodansky, A. Hosp. for Joint Diseases, N.Y.
Bodansky, Oscar Edgewood Arsenal, Md.
Bodine, J. H. State Univ. of Iowa
Bogen, Emil Olive View, Calif.
Boissevain, Charles H. Colorado Coll.
Bollman, Jesse L. Mayo Clinic
Booher, Lela E. Minneapolis, Minn.
Boor, Alden K. Univ. of Chicago
Boothby, Walter M. Mayo Clinic, Rochester, Minn.
Boots, Ralph H. Presbyterian Hosp., N. Y.
Bornstein, S. Jewish Hosp., Brooklyn
Borsook, Henry Calif. Inst. of Technology
Bowen, B. D. Buffalo Gen. Hosp.
Bowman, D. E. Indiana Univ. Med.
Boyce, F. F. New Orleans, La.
Boyd, Eldon M. Queens Univ., Canada
Boyd, Theo. E. Loyola Univ.
Boyden, E. A. Univ. of Minn. Med.
Bozler, Emil Ohio State Univ.
Bradbury, J. T. Univ. of Iowa Med.
Bradford, William L. Univ. of Rochester
Bradley, H. C. Univ. of Wisconsin
Brand, Erwin Coll. Phys. and Surg., N. Y.
Branham, Sara E. Nat. Inst. of Health
Braun-Menendez, E. Univ. of Buenos Aires
Brazda, F. G. Louisiana State Univ.
Brewer, George Univ. of Pa.
Briggs, A. P. Univ. of Georgia
Brinkhous, K. M. Univ. of Iowa
Brodie, B. B. Goldwater Mem. Hosp., N. Y.
Broh-Kahn, R. H. May Inst. Med. Research, Cincinnati
Bronfenbrenner, J. Washington Univ.

Bronk, D. W. — Univ. of Penn.
Brookhart, J. M. — Loyola Med.
Brooks, Clyde — Essex Coll. of Med.
Brooks, C. M. — Johns Hopkins Univ.
Brooks, Matilda M. — Univ. of Calif.
Brooks, S. C. — Univ. of Calif.
Broun, G. O. — St. Louis Univ. Med.
Brown, Frank A., Jr. — Northwestern Univ.
Brown, J. Howard — Johns Hopkins Univ.
Brown, John B. — Ohio State Univ.
Brown, Rachel — N. Y. State Dept. of Health
Browne, J. S. L. — Royal Victoria Hosp., Montreal
Brozek, J. — Univ. of Minn.
Brues, Austin M. — Univ. of Chicago
Bruger, Maurice — N. Y. Post-Graduate Med.
Brunschwig, Alexander — Univ. of Chicago
Bryan, W. Ray — National Cancer Inst.
Buchanan, A. R. — Univ. of Colo. Med.
Buchanan, Robert E. — Iowa State Coll.
Buchbinder, W. C. — Michael Reese Hosp., Chicago
Bucy, Paul C. — Univ. of Illinois Med.
Bueding, Ernest — Western Reserve Univ.
Buell, Mary V. — Johns Hopkins Univ.
Bukantz, S. C. — Army Medical School
Bulger, H. A. — Washington Univ.
Bunney, W. E. — New Brunswick, N. J.
Buño, Washington — Fac. Med. Montevideo
Bunting, C. H. — Hamden, Conn.
Burch, George E. — New Orleans, La.
Burch, John C. — Vanderbilt Univ. Med.
Burdon, Kenneth L. — Baylor Univ. Med.
Burk, Dean — National Cancer Inst.
Burky, Earl L. — Johns Hopkins Hosp.
Burn, C. G. — Long Island Coll. of Med.
Burns, E. L. — Louisiana State Univ.
Burns, Robert K., Jr. — Carnegie Inst., Baltimore, Md.
Burr, George O. — Univ. of Minn.
Burrows, M. T. — Pasadena, Calif.
Burrows, William — Univ. of Chicago
Burstein, C. L. — N. Y. Univ. Med.
Butcher, E. O. — N. Y. Univ. Dental Coll.
Butler, E. G. — Princeton Univ.
Butt, E. M. — Univ. of So. Calif.
Butts, Joseph S. — Oregon State Coll.
Byerly, T. C. — U. S. Animal Exp. Farm, Beltsville, Md.

Cahill, W. M. — Wayne Univ.
Calder, R. M. — San Antonio, Tex.
Calvin, D. Bailey — Univ. of Texas Med.
Cameron, A. T. — Univ. of Manitoba
Cameron, H. S. — Univ. of Calif.
Cameron, John A. — Dallas, Texas
Campbell, Berry — Univ. of Minn.
Campbell, D. H. — Cal. Inst. of Tech.
Campos, F. A. deM. — Univ. of Sao Paulo
Cannan, Robert K. — N. Y. Univ.
Cannon, Paul R. — Univ. of Chicago
Cantarow, Abraham — Jefferson Med. Coll.
Cantoni, G. L. — Long Island Coll. of Med.
Carey, E. J. — Marquette Univ.
Carlson, A. J. — Univ. of Chicago
Carmichael, E. B. — Univ. of Alabama Med.
Carmichael, L. — Tufts Coll.
Carpenter, C. M. — Univ. of Rochester
Carr, C. J. — Univ. of Md.
Carr, J. L. — Univ. of Calif. Hosp.
Cartland, G. F. — Kalamazoo, Mich.
Carruthers, A. — Birmingham, England
Cary, C. A. — U. S. Dept. of Agriculture
Casals, Jorge — Rockefeller Inst.
Casey, Albert E. — Birmingham, Ala.
Casida, L. E. — Univ. of Wisconsin
Castaneda, M. R. — Hosp. General, Mexico City
Castle, W. B. — Boston City Hosp.
Cattell, McKeen — Cornell Univ. Med. Coll.
Cecil, R. L. — Cornell Univ. Med. Coll.
Cerecedo, L. R. — Fordham Univ.
Chadwicks, C. S. — Vanderbilt Univ.
Chagas, Carlos — Univ. of Brazil
Chaikoff, I. L. — Univ. of Calif.
Chambers, Robert — New York Univ.
Chambers, Wm. H. — Cornell Univ. Med. Coll.
Chang, Hsi Chun — Peiping Union Med. Coll.
Chang, Hsiao-Chien — Hunan, China
Chargaff, Erwin — Coll. of Phys. and Surg.
Charipper, H. A. — New York Univ.
Chase, M. W. — Rockefeller Inst.
Cheer, S. N. — W. China Union Univ
Chen, Graham M. — Univ. of Chicago
Chen, K. K. — Eli Lilly and Co., Indianapolis
Chen, T. T. — Peiping Union Med. Coll.
Cheney, R. H. — Long Island Univ.
Cheyne, J. D. — State Univ. of Iowa Dent.
Chidester, F. E. — Newark Valley, N. Y.
Child, C. M. — Stanford Univ.
Chouke, K. S. — Univ. of Pa.
Chow, B. F. — New Brunswick, N. J.
Christensen, K. — St. Louis Univ.
Christian, Henry A. — Harvard Univ.
Christman, Adam A. — Univ. of Mich.
Chu, F. T. — Peiping Union Med. Coll.
Chung, H. L. — Peiping Union Med. Coll.
Clark, Ada R. — Coll. of Phys. and Surg.
Clark, George — Orange Park, Fla.
Clark, Guy W. — Lederle Lab., Pearl River, N.Y.
Clark, P. F. — Univ. of Wisconsin
Clark, W. G. — Univ. of S. Calif.
Clarke, Hans T. — Coll. of Phys. and Surg.

Claude, A. Rockefeller Inst.
Clausen, H. J. Loyola Dental School, La.
Claussen, S. W. Strong Memorial Hosp., Rochester, N. Y.
Clawson, Benjamin J. Univ. of Minn.
Clifton, Charles E. Stanford Univ.
Climenko, D. R. Rensselaer, N. Y.
Clowes, G. H. A. Eli Lilly & Co., Indianapolis
Code, C. F. Mayo Foundation
Coggeshall, L. T. Univ. of Mich.
Cohen, Barnett Johns Hopkins Med.
Cohen, Milton B. St. Alexis Hosp., Cleveland
Cohn, A. E. Rockefeller Inst., N. Y.
Cohn, David J. Michael Reese Hosp., Chicago
Cohn, Isidore New Orleans, La.
Cole, Arthur G. Univ. of Ill. Med.
Cole, Harold H. Univ. Calif., Davis
Cole, L. J. Univ. of Wisconsin
Cole, Rufus I. Rockefeller Inst., N. Y. City
Cole, Warren H. Univ. of Ill. Med.
Cole, William H. Rutgers Univ.
Colien, F. E. Creighton Univ.
Collens, William S. Brooklyn, N. Y.
Collier, William D. St. Elizabeth's Hosp., Youngstown, O.
Collings, W. D. Univ. of Texas Med.
Collins, D. A. Temple Univ. Med.
Collip, J. B. McGill Univ.
Compere, E. L. Northwestern Med.
Conant, N. F. Duke Univ.
Cook, Charles A. Boston, Mass.
Cook, Donald H. Univ. of Miami
Cook, E. S. Cincinnati, O.
Cooke, J. V. Washington Univ.
Cooper, Frank B. Chicago, Ill.
Cooper, Merlin L. Univ. of Cincinnati
Cope, O. M. N. Y. Med. Coll.
Copenhaver, W.M. Coll. Phys. and Surg., N.Y.
Corbin, Kendall B. Mayo Clinic
Corey, E. L. Univ. of Va.
Cori, Carl F. Washington Univ.
Corley, Ralph C. Purdue Univ.
Corner, George W. Carnegie Inst., Baltimore
Corper, H. J. Nat. Jewish Hosp., Denver, Col.
Co Tui N. Y. Univ. Med. Coll.
Coulson, E. J. U. S. Dept. of Agriculture
Cowdry, E. V. Washington Univ.
Cowgill, George R. Yale Univ.
Cox, A. J., Jr. Stanford Univ. Med.
Cox, Herald R. Pearl River, N. Y.
Cox, Warren M., Jr. Evansville, Indiana
Cram, Eloise B. Nat. Inst. of Health
Crampton, C. Ward N. Y. Post-Graduate Med.
Crandall, L. A., Jr. Univ. of Tenn.
Creaser, C. W. Wayne Univ. Med.
Crittenden, Phoebe J. Westfield, N. J.
Crohn, Burrell B. Mt. Sinai Hosp., N. Y.
Crossley, M. L. Bound Brook, N. J.
Croxatto, H. Catholic Univ., Chile
Cruz, W. O. Rio de Janeiro, Brazil
Cruz-Coke, E. Univ. of Santiago, Chile
Csonka, F. A. U. S. Dept. of Agriculture
Cullen, Stuart C. State Univ. of Iowa Med.
Culler, E. A. Univ. of Rochester
Cummins, Harold Tulane Univ.
Curtis, G. M. Ohio State Univ.
Curtis, Maynie R. Mason, Mich.
Cutler, Elliott C. Peter Bent Brigham Hosp., Boston
Cutting, R. A. Georgetown Univ. Med.
Cutting, W. C. Stanford Univ. Med.
Cutuly, Eugene Clairton, Pa.
Cuyler, W. K. Duke Univ.

Dack, Gail M. Univ. of Chicago
Daft, F. S. Nat. Inst. of Health
Dakin, H. D. Ossining, N. Y.
Dalldorf, Gilbert N. Y. State Dept. of Health, Albany
Dalton, A. J. National Cancer Inst.
Dam, Henrik Univ. of Rochester Med.
D'Amour, F. E. Univ. of Denver
Danforth, Charles H. Stanford Univ.
Danforth, D. N. Evanston, Ill.
Daniels, Amy L. Iowa City, Iowa
Daniels, Troy C. Univ. of Calif.
Danzer, Charles S. N. Y. Med. Coll.
Darby, E. M. K. Pelham Manor, N. Y.
Dautrebande, Lucien Univ. of Liege, Belgium
Davenport, H. W. Univ. of Utah Med.
Davidsohn, Israel Rush Med. Coll., Chicago
Davis, H. A. Coll. of Med. Evangelists, Calif.
Davis, J. E. Univ. of Arkansas
Davis, M. E. Univ. of Chicago
Dawson, James A. Coll. City of New York
Day, A. A. Northwestern Univ. Med.
Day, Harry G. Indiana Univ.
Day, Paul L. Univ. of Arkansas Med.
DeBakey, M. E. Tulane Univ.
DeBodo, Richard New York Univ. Med.
Decherd, George M. Univ. of Texas Med.
DeEds, Floyd U. S. Dept. of Agriculture
DeFries, R. D. Univ. of Toronto
DeGara, P. F. Cornell Univ. Med.
DeGowin, E. L. State Univ. of Iowa
DeGraff, A. C. N. Y. Univ. Med.
del Castillo. E. B. Univ. of Buenos Aires
Dempsey, E. W. Harvard Univ. Med.
Dennis, Clarence Univ. of Minn. Med.
De Savitsch, Eugene Washington, D. C.
Detwiler, S. R. Columbia Univ.
Deuel, Harry J., Jr. Univ. of S. Calif. Med.

Deulofeu, V. Univ. of Buenos Aires
Dey, F. L. Northwestern Univ. Med.
Dick, George F. Univ. of Chicago
Dieckmann, W. J. Univ. of Chicago
Dienes, Louis Mass. Gen. Hosp., Boston
Dieuaide, Francis R. Harvard Univ.
Dille, J. M. Univ. of Illinois
Dingle, J. H. Boston City Hosp.
Doan, Charles A. Ohio State Univ.
Dobriner, K. Memorial Hosp., N.Y.C.
Dochez, A. R. Presbyterian Hosp., N.Y.C.
Dock, William New York City
Dohan, F. C. Univ. of Pa.
Doisy, Edward A. St. Louis Univ.
Dolley, W. L., Jr. Univ. of Buffalo
Dominguez, R. St. Luke's Hosp., Cleveland
Domm, L. V. Univ. of Chicago
Donahue, J. K. Coll. of Charleston, S. C.
Donaldson, J. C. Univ. of Pittsburgh
Dorfman, A. Beth Israel Hosp., Boston
Dorfman, Ralph I. Western Reserve Univ.
Dotti, L. B. St. Luke's Hosp., N. Y. City
Doubilet, Henry Mt. Sinai Hosp., N. Y.
Doull, J. A. Western Reserve Univ.
Dounce, A. L. Univ. of Rochester
Dow, R. S. Univ. of Oregon
Doyle, William L. Univ. of Chicago
Drabkin, D. L. Univ. of Pa.
Dragstedt, Carl A. Northwestern Univ.
Dragstedt, Lester R. Univ. of Chicago
Draper, William B. Univ. of Colo. Med.
Dreisbach, R. A. Stanford Univ. Med.
Drennan, A. M. Univ. of Edinburgh
Dresbach, M. Hahnemann Med. Coll.
Drill, V. A. Yale Univ. Med.
Drury, D. R. Univ. of S. Calif. Med.
Dubin, Harry E. N. Y. City
DuBois, E. F. Cornell Univ. Med. Coll.
DuBois, F. S. Hartford, Conn.
Dubos, Rene J. Rockefeller Inst.
Dukes, H. H. Cornell Univ.
Dunihue, F. W. Univ. of Vt. Med.
Dunlap, C. E. Tulane Univ.
Dunn, Leslie C. Columbia Univ.
Dunn, Max Univ. of Calif., L. A.
Duran-Reynals, F. Yale Univ.
Dutcher, R. Adams Penn State Coll.
Du Vigneaud, Vincent Cornell Univ. Med. Coll.
Dye, Joseph A. Cornell Univ. Med. Coll.
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Eddy, Walter H. New York City
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Edwards, J. G. Univ. of Buffalo
Edwards, Philip R. Kentucky Agri. Exp. Sta.
Eggston, Andrew A. N. Y. Manhattan Eye, Ear Hosp.
Eichelberger, Lillian Univ. of Chicago
Eiler, John J. Univ. of Calif.
Ellinger, F. P. Long Island Coll. of Med.
Ellis, Max M. Univ. of Mo.
Ellis, N. R. U. S. Dept. of Agriculture
Elman, R. Washington Univ. Med.
Elser, W. J. Cornell Univ. Med. Coll.
Elvehjem, C. A. Univ. of Wisconsin
Emerson, George A. Univ. of Texas Med.
Emerson, Gladys A. Rahway, N. J.
Emerson, Sterling Calif. Inst. of Technology
Emery, F. E. Univ. of Ark.
Emge, L. A. Stanford Univ.
Emmart, E. W. Nat. Inst. of Health
Enders, J. F. Harvard Univ.
Engle, E. T. Coll. Phys. and Surg., N.Y.
Ensworth, H. K. Cornell Univ.
Epstein, A. A. Beth Israel Hosp., N. Y.
Erlanger, Joseph Washington Univ.
Ernstene, Arthur C. Cleveland Clinic
Ershoff, B. H. Los Angeles, Calif.
Essex, Hiram E. Mayo Clinic
Estable, C. Inst. of Biol., Montevideo
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Evans, Chas. A. Univ. of Minn. Med.
Evans, Earl A., Jr. Univ. of Chicago
Evans, Gerald T. Univ. of Minn.
Evans, Herbert M. Univ. of Calif.
Evans, T. C. Coll. of Phys. and Surg., N. Y.
Everett, M. R. Univ. of Okla. Med.
Ewing, P. L. Univ. of Texas Med.
Eyster, J. A. E. Univ. of Wisconsin

Faber, Harold K. Stanford Univ. Med.
Falk, K. George N. Y. Univ. Med. Coll.
Famulener, L. W. Englewood, N. J.
Farmer, Chester Northwestern Univ.
Farr, L. E. Rockefeller Inst.
Fashena, G. J. Southwestern Med. Coll.
Fassett, D. W. Miami, Fla.
Faust, Ernest C. Tulane Univ.
Favorite, G. O. Hahnemann Med. Coll.

Fearing, Franklin — Univ. of Calif., L. A.
Feil, Harold — Western Reserve Univ.
Feinstone, W. H. — Yonkers, N. Y.
Feller, A. E. — Ft. Bragg, N. C.
Fellows, E. J. — Temple Univ.
Feng, T. P. — Rockefeller Inst.
Fenn, Wallace O. — Univ. of Rochester Med.
Fenning, C. — Univ. of Utah Med.
Ferguson, John H. — Univ. of North Carolina
Ferguson, J. K. W. — Univ. of Toronto
Farraro, Armando — N. Y. S. Psychiatric Inst.
Ferry, R. M. — Harvard Univ.
Fevold, Harry L. — West Reg. Res. Lab., Albany, Calif.
Field, John, II — Stanford Univ.
Field, John B. — Univ. of Rochester Med.
Figge, F. H. J. — Univ. of Maryland Med.
Findley, T. P. — New Orleans, La.
Fine, Jacob — Beth Israel Hosp., Boston
Fine, M. S. — Hoboken, N. J.
Finerty, John C. — Univ. of Mich.
Finland, M. — Boston City Hosp.
Firor, W. M. — Johns Hopkins Univ.
Fischer, Albert — Copenhagen, Denmark
Fischer, E. — Med. Coll. of Va.
Fischer, Martin H. — Univ. of Cincinnati
Fishberg, Ella H. — Beth Israel Hosp., N. Y.
Fisk, R. T. — Pasadena, Calif.
Fitzhugh, O. G. — Food and Drug Admin.
Fleisher, Moyer S. — St. Louis Univ.
Flexner, James — N. Y. Post-Graduate Med.
Fluhmann, Charles F. — Stanford Univ. Med.
Foa, P. A. — Chicago Med. School
Foglia, V. G. — Univ. of Buenos Aires
Foley, James O. — Univ. of Alabama
Fontaine, T. D. — U. S. Dept. of Agriculture
Forbes, Henry — Milton, Mass.
Forbes, John C. — Med. Coll. of Va.
Forkner, Claude E. — N. Y. City
Forster, G. F. — Dept. of Public Health, Illinois
Fortuyn, A. B. D. — The Hague, Netherlands
Foster, R. H. K. — St. Louis Univ. Med.
Fowler, Willis M. — State Univ. of Iowa
Fox, Charles L., Jr. — Coll. of Phys. and Surg.
Fox, D. L. — Scripps Inst., Calif.
Fraenkel-Conrat, H. — Univ. of Calif.
Francis, Thomas, Jr. — Univ. of Mich.
Frank, Robert T. — Mt. Sinai Hosp.
Franke, F. E. — St. Louis Univ.
Fraps, R. M. — U. S. Dept. of Agri.
Frazier, Chester N. — Univ. of Texas Med.
Free, A. H. — Western Reserve Univ.
Freed, S. C. — Mt. Zion Hosp., San Francisco
Freedlander, B. L. — Mt. Zion Hosp., Calif.
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Freedman, Louis — N. Y. City
Freeman, R. G., Jr. — Boston, Mass.
Freudenberger, C. B. — Univ. of Utah Med.
Freund, Jules — Bureau of Laboratories, N.Y.C.
Fridericia, L. S. — Univ. of Copenhagen, Denmark
Friedemann, T. E. — Northwestern Univ. Med.
Friedewald, W. F. — Emory Univ. Med.
Friedman, M. H. — Washington, D. C.
Friedman, M. H. F. — Jefferson Med. Coll.
Friedman, M. W. — Mt. Zion Hosp., San Francisco
Fritz, James C. — Elgin, Ohio
Frobisher, Martin, Jr. — Baltimore, Md.
Fugo, N. W. — Lombard, Ill.
Fuhrman, F. A. — Stanford Univ.
Fulton, John F. — Yale Med.
Fulton, M. — Southwestern Med. Coll.
Funk, Casimir — N. Y. City
Furth, Jacob — Cornell Univ. Med. Coll.

Gaebler, O. H. — Henry Ford Hosp., Detroit
Gaffron, H. — Univ. of Chicago
Gall, Edward A. — Bethesda Hosp., Cincinnati, O.
Gallagher, T. F. — Univ. of Chicago
Gamble, James L. — Harvard Univ.
Garbat, Abraham L. — Lenox Hill Hosp., N. Y.
Gardner, Leroy U. — Saranac Lab. Tuberculosis
Gardner, William U. — Yale Univ.
Gaskell, John F. — Cambridge, Eng.
Gasser, Herbert S. — Rockefeller Inst.
Gassner, Francis X. — Colorado State Coll. Agr.
Gates, W. H. — Louisiana State Univ.
Gatz, A. J. — Loyola Univ. Med.
Gaunt, Robert — New York Univ.
Gebhardt, L. P. — Univ. of Utah Med.
Geiger, A. J. — Yale Univ. Med.
Geiger, E. — Los Angeles, Calif.
Geiling, E. M. K. — Univ. of Chicago
Gelarie, Arnold J. — Ossining, N. Y.
Gelfan, Samuel — Coll. Phys. and Surg., N.Y.
Gellhorn, Alfred — Coll. Phys. and Surg., N. Y.
Gellhorn, Ernst — Univ. of Minn.
Gengerelli, J. A. — Washington, D. C.
Georgi, C. E. — Univ. of Nebraska
Gerard, R. W. — Univ. of Chicago
Gersh, I. — Johns Hopkins Med.
Gerstenberger, H. J. — Western Reserve Univ.
Gerstl, B. — Yale Univ.
Gesell, Robert A. — Univ. of Mich.
Gettler, A. O. — N. Y. Univ. Med. Coll.
Gibbs, O. S. — Memphis, Tenn.
Gibson, R. B. — State Univ. of Iowa
Gies, William J. — Coll. Phys. and Surg., N.Y.
Gilligan, Dorothy R. — Cornell Univ. Med.
Gilman, Alfred — Yale Univ.

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Girden, Edward Brooklyn Coll.
Givens, Maurice H. Chicago, Ill.
Glaser, Otto C. Amherst Coll.
Glass, S. J. Los Angeles, Calif.
Glasser, Otto Cleveland Clinic
Glaubach, Susi Beth Israel Hosp., Newark
Glazko, A. J. Emory Univ.
Glick, D. Minneapolis, Minn.
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Gold, Harry Cornell Univ. Med. Coll.
Goldberg, S. A. Presbyterian Hosp., Newark
Goldblatt, Harry Western Reserve Univ.
Goldfarb, Walter Bellevue Hosp., N. Y.
Goldfeder, Anna New York Univ.
Goldforb, A. J. Coll. City of New York
Goldie, Horace Nanuet, N. Y.
Goldner, M. G. Univ. of Chicago Med.
Goldring, W. N. Y. Univ. Med.
Goldschmidt, Samuel Univ. of Pa.
Goldsmith, E. D. N. Y. U. Med.
Gomori, George Univ. of Chicago
Goodman, L. Univ. of Utah
Goodner, Kenneth Uganda, E. Africa
Gordon, A. S. Washington Sq. Coll.
Gordon, F. B. Univ. of Chicago
Goss, C. M. Birmingham, Ala.
Goss, Harold Univ. of Calif.
Grace, A. W. Long Island Coll. of Med.
Graef, Irving N. Y. Univ. Med. Coll.
Graeser, James B. Univ. of Calif.
Graham, Evarts A. Washington Univ.
Graham, Helen T. Washington Univ.
Gray, John S. Northwestern Univ. Med.
Gray, Samuel H. Jewish Hosp., St. Louis, Mo.
Gray, S. J. Univ. of Chicago
Greeley, P. O. Univ. of S. Calif.
Green, Harold D. Bowman Gray School of Med.
Green, Robert G. Univ. of Minn.
Greenberg, David M. Univ. of Calif.
Greenberg, L. D. Univ. of Calif. Med.
Greene, Carl H. Brooklyn, N. Y.
Greene, Harry S. N. Yale Univ.
Greene, James A. Baylor Univ. Med.
Greenwald, Isidor N. Y. Univ. Med. Coll.
Greenwood, Alan Univ. of Edinburgh
Greenwood, D. A. Univ. of Chicago
Greep, R. O. Harvard Univ.
Gregersen, Magnus I. Coll. Phys. and Surg.
Gregg, D. E. Western Reserve Univ.
Gregory, Louise H. Barnard Coll.
Gregory, Paul W. Univ. of Calif.
Gregory, R. L. Univ. of Texas Med.
Griffith, Fred R., Jr. Univ. of Buffalo
Griffith, John Q., Jr. Univ. of Pa.
Griffith, Wendell H. St. Louis Univ.
Grimson, K. S. Duke Univ.
Groat, R. A. Bowman Gray Med. School
Grollman, A. Southwestern Med. Coll.
Gross, Erwin G. State Univ. of Iowa
Gross, Ludwik Cincinnati, Ohio
Gross, Paul W. Penn. Hosp., Pittsburgh
Gruber, Charles M. Jefferson Med. Coll.
Gudernatsch, F. New York Univ.
Guerrant, N. B. Penn State Coll.
Guest, G. M. Children's Hosp., Cincinnati, O.
Gunn, Francis D. Univ. of Utah
Gurchot, Charles Univ. of Calif. Med.
Gustavson, R. G. Univ. of Chicago
Gustus, E. L. Chicago, Ill.
Guthrie, C. C. Univ. of Pittsburgh
Gutierrez-Noriega, C. Univ. San Marcos, Peru
Gutman, Alexander B. Presbyterian Hosp., N.Y.
Guyer, Michael F. Univ. of Wisconsin
György, Paul Univ. of Pennsylvania

Haag, Harvey B. Med. Coll. of Va.
Haagen-Smit, A. J. Calif. Inst. of Tech.
Hac, Lucile R. Woodland, Calif.
Haden, Russell L. Cleveland Clinic
Hadley, Philip W. Penn. Hosp., Pittsburgh
Hafkesbring, H. Roberta Woman's Med. Coll., Philadelphia
Hagan, William Arthur Cornell Univ.
Haig, Charles N. Y. Med. Coll.
Halberstaedter, L. Hebrew Univ., Jerusalem
Hale, William M. State Univ. of Iowa
Hall, Ernest M. Univ. of S. Calif. Med.
Hall, I. C. Portland, Ore.
Hall, R. P. New York Univ.
Hall, V. E. Stanford Univ.
Halliday, Nellie Univ. of Calif.
Halpert, Béla Univ. of Oklahoma Med.
Halsey, Robert H. N. Y. Post-Graduate Med.
Hambourger, Walter E. Chicago, Ill.
Hamilton, B. K. Chicago, Ill.
Hamilton, J. B. Long Island Coll. of Med.
Hammer, B. W. Iowa State Coll.
Hammon, W. McD. Univ. of Calif.
Hampel, C. W. New York Univ. Med.
Handler, Philip Duke Univ. Med.
Handley, C. A. Baylor Univ. Med.
Haney, H. F. Univ. of Wisconsin
Hanger, Franklin M. Presbyterian Hosp., N. Y. City
Hanke, Martin E. Univ. of Chicago
Hanks, J. H. Culion, Philippine Islands
Hansen, Arild E. Univ. of Texas Med.
Hansmann, G. H. Columbia Hosp., Milwaukee
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Hanzlik, P. J. Stanford Univ. Med.

Hardy, J. D. — Cornell Med. Coll.
Hare, Kendrick — State Univ. of Iowa
Harkavy, Joseph — Mt. Sinai Hosp., N. Y.
Harkins, Henry N. — Johns Hopkins Univ.
Harned, Ben K. — Pearl River, N. Y.
Harris, A. S. — Western Reserve Univ.
Harris, Isaac F. — Rye, N. Y.
Harris, Meyer M. — N. Y. State Psychiatric Inst.
Harris, Philip L. — Rochester, N. Y.
Harris, William H. — Tulane Univ.
Harrison, R. G. — Yale Univ.
Harrison, Roland W. — Univ. of Chicago
Harrison, T. R. — Southwestern Med. Coll.
Harrow, Benjamin — Coll. City of New York
Hart, Edwin B. — Univ. of Wisconsin
Hart, George H. — Univ. Farm, Davis, Calif.
Hartman, F. A. — Ohio State Univ.
Hartman, F. W. — Ford Hosp., Detroit
Harvey, E. Newton — Princeton Univ.
Harvey, Samuel C. — Yale Univ.
Hastings, A. Baird — Harvard Univ.
Haterius, H. O. — Boston Univ. Med.
Hathaway, Edward S. — Tulane Univ.
Haury, V. G. — Audubon, N. J.
Hawk, P. B. — New York City
Hawkins, J. E., Jr. — Bowman Gray School of Med.
Hayden, Charles E. — Cornell Univ.
Hayman, J. M., Jr. — Western Reserve Univ.
Haythorn, Samuel R. — Singer Research Lab., Pittsburgh, Pa.
Hays, E. E. — Univ. of Vermont Med.
Hays, Harry W. — Summit, N. J.
Hazleton, L. W. — George Washington Univ.
Hecht, Hans H. — Univ. of Utah Med.
Heckel, N. J. — Univ. of Chicago
Hegnauer, Albert H. — Syracuse Univ.
Hehre, E. J. — Cornell Univ. Med.
Heidelberger, Michael — Presbyterian Hosp., N.Y.
Heilbrunn, L. V. — Univ. of Pa.
Heilman, Dorothy H. — Mayo Clinic
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Heilman, J. — Coll. of Phys. and Surg., N.Y.
Heinbecker, Peter — Washington Univ.
Heinle, R. W. — Western Reserve Univ.
Helff, O. M. — New York Univ.
Hellbaum, Arthur A. — Univ. of Okla.
Hellebrandt, F. A. — Richmond, Va.
Heller, C. G. — Univ. of Oregon Med.
Helmer, O. M. — Indiana Univ.
Helmholz, Henry F. — Mayo Clinic
Hemingway, A. — Univ. of Minn.
Hendrix, B. M. — Univ. of Texas
Henle, Werner — Univ. of Pennsylvania
Henschel, A. — Univ. of Minn.
Hepler, Opal E. — Northwestern Univ. Med.
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Herrold, Russell D. — Univ. of Ill. Med.
Hershey, A. D. — Washington Univ.
Hertz, Roy — Nat. Inst. of Health
Hertzman, A. B. — St. Louis Univ.
Herwick, Robert P. — Georgetown Univ.
Hess, Walter C. — Georgetown Univ.
Hess Walter N. — Hamilton Coll.
Hesseltine, H. C. — Univ. of Chicago
Hetherington, D. C. — Duke Univ. Med.
Hewitt, E. A. — Iowa State Coll.
Heymann, Walter — Western Reserve Univ.
Heymans, J. C. — U. de Gand, Belgium
Hiestand, W. A. — Purdue Univ.
Hill, F. C. — Creighton Univ. Med.
Hill, Robert M. — Univ. of Colo. Med.
Himwich, H. E. — Albany Med. Coll.
Hines, H. M. — State Univ. of Iowa
Hinman, E. H. — T. V. A., Ala.
Hinrichs, Marie Agnes — Carbondale, Ill.
Hinsey, J. C. — Cornell Med. Coll.
Hinshaw, H. C. — Mayo Clinic
Hirshfeld, J. W. — Cornell Univ.
Hirst, G. K. — Rockefeller Foundation
Hisaw, F. L. — Harvard Univ.
Hitchcock, Fred A. — Ohio State Univ.
Hitchings, G. H. — Tuckahoe, N. Y.
Hoagland, C. L. — Rockefeller Inst.
Hobby, Gladys L. — Brooklyn, N. Y.
Höber, Rudolf — Univ. of Pa.
Hobmaier, Michael — Univ. of Calif.
Hodge, H. C. — Univ. of Rochester
Hoeppli, R. J. C. — Peiping Union Med. Coll.
Hoerr, N. L. — Western Reserve Univ.
Hoffman, W. S. — Chicago Med.
Hogan, Albert G. — Univ. of Mo.
Holck, H. G. O. — Univ. of Nebraska
Hollaender, A. — National Inst. of Health
Hollander, Franklin — Mt. Sinai Hosp., N. Y.
Hollander, W. F. — New Haven, Conn.
Holm, George E. — Dept. Agr., Washington, D. C.
Holman, Emil — Stanford Univ.
Holman, R. L. — Univ. of N. Carolina
Holman, W. L. — Univ. of Toronto
Holt, L. Emmett, Jr. — New York Univ.
Hooker, Charles W. — Yale Univ. Med.
Hooker, Sanford B. — Boston Univ.
Hopkins, J. Gardner — Coll. Phys. and Surg., N.Y.
Horsfall, F. L., Jr. — Rockefeller Foundation
Horsley, J. Shelton — St. Elizabeth's Hosp., Richmond, Va.
Horwitt, M. K. — Elgin, Ill.
Hoskins, William M. — Univ. of Calif.
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Howitt, Beatrice F. U. S. Public Health, Ala.
Hoyt, Anson P. S. Univ. of So. Calif.
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Hu, C. K. Peiping Union Med. Coll.
Hubbard, Roger S. Buffalo Gen. Hosp.
Huddleston, Ora L. Univ. of Colo. Med.
Hudson, N. Paul Ohio State Univ.
Huffman, J. W. Northwestern Univ. Med.
Hug, Enrique Universidad Nacional, Rosario
Huggins, C. B. Univ. of Chicago
Hughes, Thomas P. Rio de Janeiro, Brazil
Humphrey, R. R. Univ. of Buffalo
Hunscher, H. A. Western Reserve Univ.
Hunt, Thomas E. Univ. of Alabama Med.
Hurtado, A. Univ. of San Marcos, Peru
Hussey, Raymond Detroit, Mich.

Ingersoll, E. H. Med. Coll. of Va.
Ingle, D. J. Kalamazoo, Mich.
Ingram, Walter R. State Univ. of Iowa
Irving, G. W., Jr. U. S. Dept. of Agriculture
Irwin, M. R. Univ. of Wisconsin
Isaacs, Raphael Michael Reese Hosp., Chicago
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Jackson, D. E. Univ. of Cincinnati
Jackson, Richard W. U. S. Dept. of Agri.
Jacobs, Henry R. Northwestern Univ. Med.
Jacobs, J. L. Oglethorpe Univ.
Jacobs, M. H. Univ. of Pa.
Jacobs, Walter A. Rockefeller Inst., N. Y.
Jacobson, L. O. Univ. of Chicago
Jaffe, Henry L. Hosp. for Joint Diseases, N.Y.
Jahn, Theodore L. State Univ. of Iowa
Jameson, Eloise Stanford Univ.
Janes, R. G. State Univ. of Iowa Med.
Jaques, L. B. Univ. of Toronto
Jeans, Philip C. Univ. of Iowa
Jennison, M. W. Mass. Inst. of Technology
Jensen, Hans F. Montreal
Jochim, K. E. Michael Reese Hosp., Chicago
Johlin, J. M. Vanderbilt Univ. Med.
Johnson, B. C. Univ. of Illinois
Johnson, C. A. Miamesberg, O.
Johnson, C. C. Grace, Idaho
Johnson, F. H. Princeton Univ.
Johnson, J. Garth Linden, N. J.
Johnson, Marvin J. Univ. of Wisconsin
Johnson, T. B. Yale Univ.
Johnston, C. G. Wayne Univ. Med.
Jolliffe, Norman H. N. Y. Univ. Med. Coll.
Jonas, Leon Univ. of Pa.
Jones, David B. U. S. Dept. of Agri.
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Jordan, H. E. Univ. of Va.
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Juhn, Mary Univ. of Maryland
Jukes, T. H. Pearl River, N. Y.
Jungeblut, Claus Coll. Phys. and Surg., N.Y.
Jungherr, E. Univ. of Connecticut

Kahn, Morton C. Cornell Med. Coll., N. Y.
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Kamm, Oliver Parke, Davis & Co., Detroit
Kaplan, Alex Mt. Zion Hosp., San Francisco
Karelitz, Samuel Mt. Sinai Hosp., N. Y.
Karsner, H. T. Western Reserve Univ.
Katz, Gerhard Colony, Va.
Katz, L. N. Michael Reese Hosp., Chicago
Katzenelbogen, S. Washington, D. C.
Katzman, Philip A. St. Louis Univ.
Kaufmann, William Northampton, Mass.
Keefer, Chester S. Boston Univ.
Keeton, Robert W. Univ. of Ill.
Keitt, G. W. Univ. of Wisconsin
Keller, Allen D. Baylor Univ. Med.
Kelly, G. L. Univ. of Georgia Med.
Kemmerer, A. R. Univ. of Arizona
Kemp, Hardy A. Wayne Univ. Med.
Kendall, E. C. Mayo Clinic, Minn.
Kendall, Forrest E. Douglaston, L. I.
Kerr, William J. Univ. of Calif. Med.
Kessel, John F. Univ. of S. Calif.
Kesten, H. D. Coll. of Phys. and Surg., N. Y.
Key, John A. Washington Univ. Med.
Keys, Ancel Univ. of Minn.
Kidd, John G. Cornell Univ. Med. Coll.
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King, Charles G. New York City
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Kleiner, I. S. N. Y. Med. Coll.
Kleitman, Nathaniel Univ. of Chicago
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Knowlton, G. C. — State Univ. of Iowa
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Kober, Philip A. — Detroit, Mich.
Koch, Fred C. — Chicago, Ill.
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Kofoid, Charles A. — Univ. of Calif.
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Kohn, Jerome L. — Mt. Sinai Hosp., N. Y.
Kolmer, John A. — Univ. of Pa.
Kopeloff, Nicholas — Psychiatric Inst., N. Y.
Koppanyi, Theodore — Georgetown Univ. Med.
Korns, Horace M. — Dubuque, Iowa
Koser, Stewart — Univ. of Chicago
Kountz, W. B. — Washington Univ.
Kozelka, F. L. — Univ. of Wisconsin
Kozoll, D. D. — Northwestern Univ. Med.
Kracke, R. R. — Med. Coll. of Ala.
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Larson, Edward — Temple Univ.
Larson, P. S. — Med. Coll. of Virginia
Larson, W. P. — Univ. of Minn.
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Levine, Philip — Linden, N. J.
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Levine, Rachmiel — Michael Reese Hosp., Chicago
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Levine, Victor E. — Creighton Univ.
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Nuckolis, James Univ. of Calif. Med.
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Peters, Lawrence Western Reserve Univ. Med.
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